Assessing the Efficacy of a Bacteriophage Cocktail, Comprised of Three Monophages, Against *Campylobacter* spp. in Broiler Chickens.

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Thesis submitted to the University of Nottingham for the degree of Master of Research

September 2024

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Keywords: *Campylobacter jejuni*, *Campylobacter coli*, Bacteriophage, Chicken liver, CampyShield, Phage cocktail, Monophage

Abstract

Campylobacter species are the leading cause of foodborne illness across the globe, with chicken meat being the main source of infection. The development of resistance, by campylobacters, to key antibiotics limits the treatment options for consumers, heightening the need to prevent transmission to humans in the first instance. This study was conducted to assess the efficacy of a bacteriophage cocktail, named CampyShield, in reducing *Campylobacter* counts in the caeca and liver of broiler chickens. The efficacy of CampyShield in *Campylobacter* reduction was compared with that of CP30A (a commonly used *Campylobacter* bacteriophage). Firstly, 160 trial birds were allocated to treatment groups, as described in section 2.6., and were infected with CampyShield, CP30A and *Campylobacter* to assess the extent of *Campylobacter* reduction in the caeca. *Campylobacter* phage-resistant isolates from the birds, were then detected, post-trial, and subjected to various resistance tests such as sensitivity testing to the CampyShield cocktail phages and CP30A, motility testing and carrier state testing. Phage-resistant isolates were also sequenced to identify phase variation, conferring resistance mechanisms. The detection of CampyShield and the presence of *Campylobacter* in the liver of trial birds was then discussed, to assess whether there was translocation by either entity from the caeca of the broiler chickens into the liver and to assess the efficacy of CampyShield in the liver. Lastly, *in vitro* investigations were carried out on the CampyShield constituent phages against *Campylobacter* strains to further determine the efficacy of the cocktail. These investigations included measuring host strain diversity, calculating efficiency of plating (EOP) values against certain strains and measuring the extent of resistance to the CampyShield constituent phages, by *C. jejuni* NCTC 12662, after 24 h. DNA sequences of these resistant strains were also obtained, to identify resistance mechanisms through phase variation.

In summary, the CampyShield cocktail lacked efficacy in several regards, due to individual drawbacks of its constituent phages. For example, CampyShield experienced a much higher resistance level in the caeca compared to that of CP30A, only one third of the constituent phages was able to translocate to the liver and CampyShield experienced a high level of resistance by *C. jejuni* NCTC 12662 lab stock isolates *in vitro*.

Acknowledgements

Firstly, I would like to offer my utmost gratitude to my supervisor, Professor Ian Connerton for allowing me to complete this Masters and for offering his continued support throughout the project. His expertise and advice have been pivotal in completing this research.

I would also like to massively extend my thanks to Dr Phillippa Connerton for her extensive support inside and outside of the laboratory, especially in times of need. She guided me through most of my laboratory practices and her contributions were vital in the completion of my project. I would not have been able to complete this research without her.

I am also grateful to my peers, Lucy Gavin and Zack Ridley, for helping me through this past year, motivating and supporting me daily and offering great advice in all aspects.

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Chapter 1- Introduction

1.1. *Campylobacter* characteristics

Campylobacter is a zoonotic pathogen that was first reported in 1913. Campylobacters are gram-negative, microaerophilic, non-spore forming bacteria that are rod-shaped, or curved, and motile, with either a single polar flagellum, bipolar flagellum or no flagellum, depending on the species. They are slender bacteria with a size range from 0.2-0.8 µm by 0.5-5 µm. Campylobacters are chemoorganotrophs that acquire energy from amino acids and perform respiratory metabolism (Kaakoush *et al*., 2015). In general, they grow optimally at temperatures ranging from 30 to 37 °C, with the exception of the thermotolerant species: *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* that grow optimally at 42 °C or between 37 to 42 °C (Mikulić *et al*., 2016). Campylobacters are fastidious in their growth requirements and are therefore sensitive to stressful conditions; *C. jejuni,* in particular, is unable to grow at temperatures below 30 °C and above 47 °C (Doyle and Roman, 1981). Campylobacters are sensitive to atmospheric oxygen and must, therefore, be stored and grown under microaerobic conditions, which typically consist of 5 % Oxygen, 5 % Hydrogen, 5 % Carbon Dioxide and 85 % Nitrogen.

1.2. *Campylobacter* taxonomy

Taxonomy is a scheme that groups organisms using three main areas: classification, identification and nomenclature (On, 2001), all linked to each other. Strains are classified based on common features and traits, which allows them to be distinguished from other strains and taxa. Major groups use cell shape, Gram stain reaction and spore formation to distinguish bacteria and the genera and species are usually determined through observing fermentation reactions, nutritional requirements and pathogenicity (Pitt and Barer, 2012). However, the mass acquisition of gene sequencing data has heralded the age of molecular taxonomy where phylogenetic relationships have become the basis for taxonomic groupings (Hugenholtz *et al*., 2021).

Portes *et al*., (2023), states that the *Campylobacter* genus is comprised of 32 species and 9 subspecies. *C. jejuni* subspecies *jejuni*, *C. jejuni* subspecies *doylei* and *C. coli*

are the most prevalent and therefore, most studied species, as they reside in humans and animal intestines. *C. lari* is also a threat to humans but is more infrequent and is generally found in seagulls. *Campylobacter* species are divided into five discrete phylogenetic groups, of which all contain pathogenic organisms. Figure 1 below shows these phylogenetic relationships.

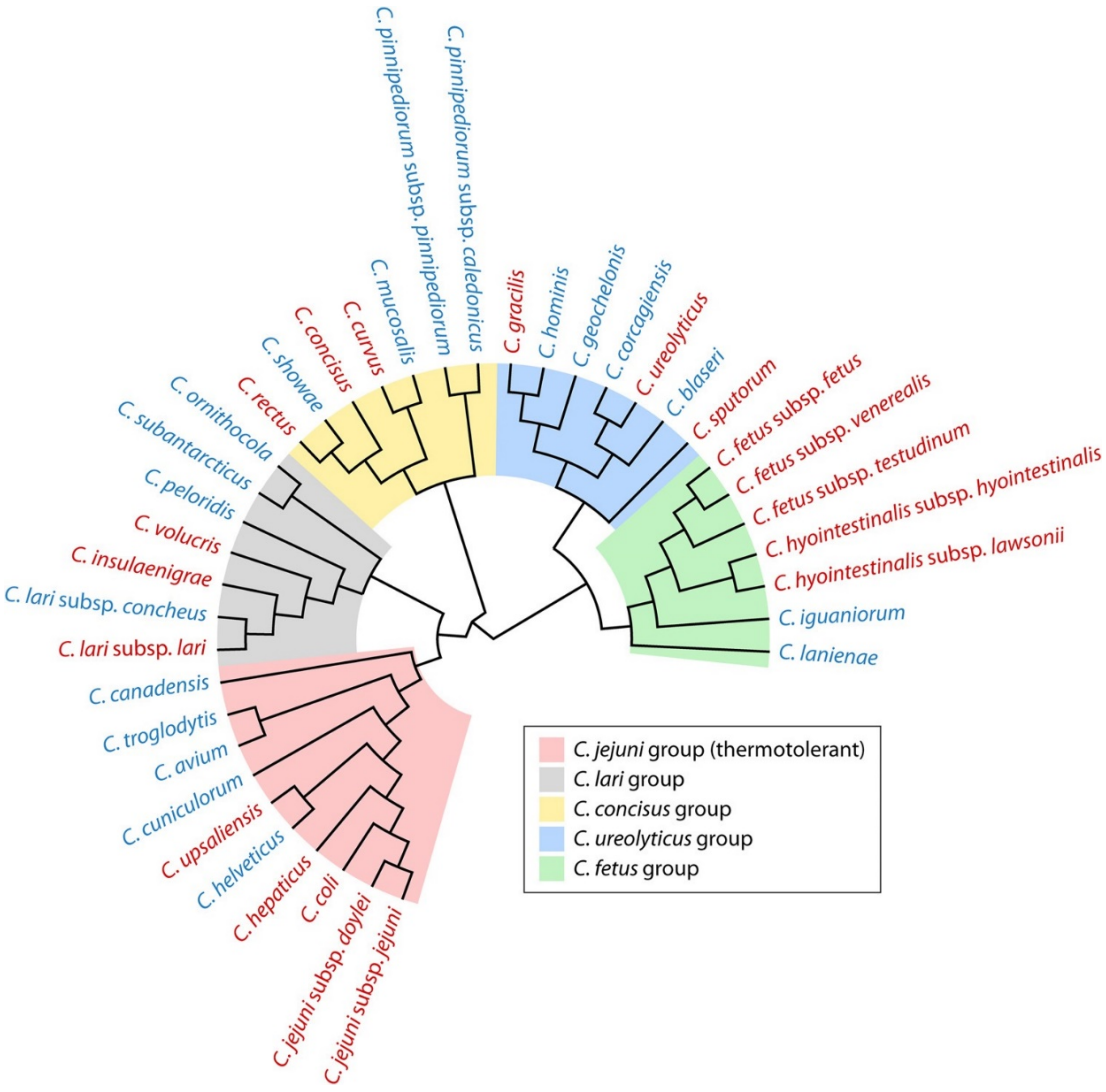

Figure 1. Phylogenetic groups and relationships between *Campylobacter* species. The five groups are named the *C. jejuni* group, *C. lari* group, *C. concisus* group, *C. ureolyticus* group and the *C. fetus* group; group names were allocated based on which species was the most relevant in clinical settings. Some campylobacters named include their subspecies (subsp.). Red labelled organisms are those that have caused infections in humans and/or animals and blue labelled species are those that have not been recorded to have caused infections (Costa and Iraola, 2019).

The genus *Campylobacter* was named in 1963 but has seen many changes since its establishment. Species determination can be carried out by comparing unspecified *Campylobacter* genomes to exact nucleotide, or k-mer, matches from the NCBI database. However, *Campylobacter* nomenclatures in this database are not based on a complete genetic or phylogenetic analysis of the entire genus. In addition, Horizontal Gene Transfer (HGT) between campylobacters presents problems when determining species within the genus as new genes with new functions are transferred among organisms so there is uncertainty between the genetic makeup of different species. Species in the same genus are often differentiated using 16S rRNA sequencing as it distinguishes bacterial genomes at these levels. However, this method may be unable to differentiate between some species such as *C. jejuni* from *C. coli*, due to the potential challenge of substitution and recombination events (Wu *et al*., 2024).

Campylobacters were initially classified in the *Vibrio* genus but studies using serological biochemical analysis and DNA base composition distinguished them from 'true' *Vibrio* spp. (On, 2001). They were finally grouped into the *Campylobacter* genus; family Campylobacteraceae, order Campylobacterales, class Epsilonproteobacteria, phylum Proteobacteria (Tion *et al*., 2024); within the Campylobacteraceae family, the *Campylobacter* genus is the largest component. '*Campylobacter pylori'* and '*Campylobacter mustelae'* were reclassified into the *Helicobacter* genus, order Campylobacterales, after numerical comparison of partial 16S rRNA sequences identified clades within *Campylobacter* and highlighted differences between these species and other *Campylobacter* spp. Differences were found in flagella structure, fatty acid and menaquinone composition and 16S rRNA gene sequences (On, 2001). Other members of this family include the genera *Sulfurospirillum* and *Arcobacter*.

1.3. *Campylobacter* pathogenesis

Campylobacter is very infectious; numbers as low as 500-800 CFU can cause health issues in humans (Tion, *et al*., 2024), such as diarrhoeal illness. Numerous studies identify *C. jejuni* as being the most prevalent species in human diseases worldwide, such as gastroenteritis, followed by *C. coli*. The pathogenic mechanism of *C. jejuni* consists of four key stages: colonisation of the digestive tract, adhesion to intestinal

cells, invasion of targeted cells and toxin production. Colonisation by *C. jejuni* occurs initially in the mucus lining the small intestine (Poly and Guerry, 2008), which is followed by adhesion, to mediate colonisation, leading to the invasion of intestinal cells. The bacteria then translocate transcellularly or paracellularly, while reproducing in the intestinal mucosa, and release toxins to kill the villi cells in the intestines (Tion, *et al*., 2024); they are subsequently able to persist in the gut, which generally manifests as diarrhoeal disease. However, campylobacterosis is a self-limiting disease with most cases resolving after 1 to 2 weeks but a few can relapse or result in complications such as irritable bowel syndrome, inflammatory bowel disease and the autoimmune diseases Guillain-Barre syndrome and reactive arthritis (WHO, 2013).

Within these stages of pathogenesis, there are several virulence factors that are crucial in the development of disease, such as, adhesion to the host cell, the role of flagella in motility and cell invasion, and lastly, toxin formation. Furthermore, successful colonisation is also based on the role of various genes, antigens and stress responses (Tion, *et al*., 2024). A common surface antigen of *C. jejuni* is flagellin (Perez-Perez and Blaser, 1996), which plays a major role in pathogenesis and adhesion. The flagellum is the source of movement for the cell and is a helical polymer made up of a flagellin protein subunit. It is rotated by the basal plate, which provides energy to propel the cell quickly through its environment. The role of the flagella in colonisation and adhesion is to enable the bacterium to reach the target site, i.e. the mucus of the intestines, by ‘chemotactically directed movements’ and to then enable adaptation to the environment by immune evasion or genetic variation (Kemper and Hensel, 2023). The flagella and the helical cell body of *C. jejuni* enable it to remain motile in highly viscous environments and move much faster in these environments than other similarly structured bacteria such as *Escherichia coli* and *Vibrio cholerae*. *E. coli* and *V. cholerae* have average velocities of 10 to 20 μm s^{-1} in an environment such as surface mucus, whereas *C. jejuni*, can maintain a velocity of around 70 μm s^{-1} in the same environment (Kemper and Hensel, 2023).

Adhesion to specific host cells is an essential step during infection by *C. jejuni.* Adhesion utilises surface components of the infecting bacterial cells, adhesins. These adhesins bind to specific proteins of the epithelium and are either ‘true’ adhesins,

which directly interact with intestinal epithelium surface receptors, or they are 'putative' adhesins, which provide support in the process. Surface proteins include CadF and FlpA, which bind to fibronectin on epithelial cells lining the intestines, and JlpA, which interacts on the host cell by binding a heat-shock protein. The binding of CadF and FlpA to fibronectin triggers intracellular signalling in the host cells, which advances cell invasion through the secretion of invasion antigens, and regulates focal adhesion, through membrane ruffling and through reducing host cell migration (Kemper and Hensel, 2023). CadF and FlpA therefore contribute to adherence, invasion and cell signalling in the colonisation process. Thus, *C. jejuni* negatively impacts host cell behaviour by changing the structure, composition and function of focal adhesins to stimulate host cell signalling and promote invasion.

Invasion of host cells by *C. jejuni* requires several mechanisms and proteins called invasins, to either cause uptake or invasion by *C. jejuni* into the intestinal epithelial cells. There are two mechanisms employed by bacteria to carry out invasion; 'zipper' and/or 'trigger' and the mechanism used depends on the proteins of the host cell cytoskeleton. The 'zipper' mechanism involves pathogen binding to host cell structures, which initiates signalling cascades that induce endocytosis. Meanwhile, the 'trigger' mechanism involves the injection of effector molecules into the host cell, which induces membrane ruffling through cytoskeletal restructuring (Kemper and Hensel, 2023). Membrane ruffling is the formation of membrane protrusions, rich in actin, that are essential for the motility of the cell and is the first step towards cell migration (Mahankali *et al*., 2011). The goal of this mechanism is to cause dramatic, localised ruffling on the host cell surface, which leads to folding of the membrane protrusions, trapping the bacterial cells in large endocytic vesicles (Alberts *et al*., 2002) and causes further host cell colonisation. Invasion of host cells is also carried out with the contribution of *Campylobacter* invasion antigens (Cia), which are exported via the flagellar Type III Secretion System (T3SS), when the bacteria come into contact with the host cells (Konkel *et al*., 2020). CiaC and CiaD are necessary for maximal invasion by *C. jejuni*. Upon invasion of the gastrointestinal tract, damaged epithelial cells are shed into the lumen at a higher frequency and toxins are released, which alter normal functioning of the cell (Friis *et al*., 2005),

Toxin production is the last stage of intestinal colonisation by *C. jejuni*, which involves the secretion of Cytolethal Distending Toxin (CDT), a holotoxin and strong virulence factor of *C. jejuni*. CDT causes the cells to become swollen, which highlights changes in ion transport regulators (Friis *et al*., 2005). This interferes with normal cell cycle progression through DNase activity that causes damage to chromosomal DNA (double-strand breaks), after translocation to the nucleus (Méndez-Olvera *et al*., 2016), where the translocation to the nucleus involves help of the cytoskeleton. The damaged cells then arrest in the growth phase of the cell cycle, which leads to apoptosis (Lee *et al*., 2003) and inflammation then bloody diarrhoea. CDT is a protein toxin, part of the DNase I protein family, and is encoded by three genes; *cdtA*, *cdtB* and *cdtC.* It is proposed that CdtA and CdtC are required for the delivery of CdtB to cells by enodcyotsis, as CdtB is the enzymatically active subunit of CDT, responsible for DNA damage and growth phase arrest. CdtA and CdtC are also responsible for interacting with the host cell membrane to enable translocation of the toxin across the membrane (Kemper and Hensel, 2023); this occurs after internalisation of extracellular CDT upon binding to a surface receptor. CDT can also induce pyroptosis in the host cell, which is an inflammatory form of programmed cell death (Gu *et al*., 2022). This study showed how CDT successfully induces pyroptosis in a dose-and-time dependent manner in human epithelial cells lining the colon. Pyroptosis is identified by cell swelling, with large bubbles emerging from the plasma membrane, along with rupturing of the cell membrane, which leads to the release of cellular contents.

1.4. *Campylobacter* sources and transmission in humans and animals

As a zoonotic and enteric pathogen, *Campylobacter* species have a broad animal reservoir and reside commensally in the small and large intestines of wild and farm animals including poultry, cattle, pigs and sheep. However, their favoured environment tends to be in the intestines of avian species, with body temperatures of 42 °C, that match optimum growth temperatures of thermophilic *Campylobacter* species. Common avian sources include wild birds, chickens, turkeys and ducks. According to numerous studies, chickens may acquire the organism through horizontal dissemination from the environment (El-Saadony *et al*., 2023). Sources of

infection may include old litter, untreated drinking water and groundwater, other farm animals or pets, rodents, farming vehicles and equipment and insects such as flies may act as vectors in the transmission of *Campylobacter* from livestock to chickens.

A study by Hald *et al*., (2004), showed that large numbers of *C. jejuni*-infected flies entered a broiler house through the ventilation systems. In July 2003, hundreds of flies per day passed through these systems into the broiler house and 8.2 % of those captured were the potential cause of *C. jejuni* spreading from outside animals to chickens in the house. Furthermore, Jacobs-Reitsma *et al*., (2009), conducted weekly screening of two Dutch poultry farms for the presence of *Campylobacter* in fresh caecal matter. The study found *Campylobacter* positive samples from darkling beetles inside the broiler houses and noted the *Campylobacter* serotypes found in these samples were identical to those isolated from the broilers. These results suggested that horizontal transmission from the environment to the birds occurred.

In Finland, a study by Hakkinen, Heiska and Hänninen (2007), investigated the prevalence of *Campylobacter* spp. in cattle, where 31 % of fecal samples and 4 % of carcass surface samples tested positive; *C. jejuni* was the most prevalent species. A reduction by 41, to 44 %, in infection rates, has been observed upon removing other livestock from chicken farms (El-Saadony *et al*., 2023). Domestic dogs and cats were also observed to harbour *Campylobacter* spp. on a regular basis and to shed *C. jejuni* and *C. coli,* and were, therefore, identified as a risk agent of infection on broiler farms. Torralbo *et al*., (2014), observed the presence of dogs and cats to be associated with increased intra-flock prevalence.

Poor hygiene habits employed by farmers around the world are also a major contribution to high *Campylobacter* incidence as uncontrolled environments increase the risk of *Campylobacter* multiplication. Bacteria can spread easily from the outside environment into the broiler farms, through various sources, but humans are a big contributing vector. Ramabu *et al*., (2004), obtained swab samples from various farming resources such as trucks, tractors, forklifts, crates, pallets and boots of workers. Upon enrichment and recovering of the samples, 53 % were found positive for *C. jejuni*; insufficient cleaning and disinfection of transportation equipment and

vehicles is likely to have caused this during transit. Therefore, it is pivotal for agricultural workers to follow thorough hygiene protocols to reduce *Campylobacter* incidence (El-Saadony *et al*., 2023).

In developing countries such as Nigeria, risk factors that promote *Campylobacter* infection are frequent as almost all households are involved in poultry or livestock production to earn a living or supply food. Moreover, in these countries, they possess poor biosecurity, contaminated water sources and poor hygiene practices on the farm and at slaughterhouses (Nwankwo, Salihu and Nwanta, 2023), which further contributes to the spread of *Campylobacter*.

According to the Minnesota Department of Health (2023), *Campylobacter* can also be found in unpasteurised milk and untreated water, so is also acquired through waterborne transmission. Water sources such as lakes, rivers, streams and coastal waters, can be contaminated through a variety of methods including direct contamination by animal and avian faeces and run-off from farms and slaughterhouses (Whiley *et al*., 2013). Table 1 outlines the sources of different *Campylobacter* species (Silva *et al*., 2018; Connerton and Connerton, 2017).

Table 1. *Campylobacter* species and several of their animal sources.

Species	**Source**
Campylobacter coli	Pigs, poultry, cattle, sheep, goats
Campylobacter concisus	Humans, dogs, cats
Campylobacter curvus	Humans and dogs
Campylobacter fetus subsp. *fetus*	Cattle, horse, sheep
Campylobacter jejuni subsp. *doylei*	Humans
Campylobacter jejuni subsp. *jejuni*	Poultry, cattle, pigs, sheep, water
Campylobacter lari subsp. *lari*	Poultry, dogs, shellfish
Campylobacter rectus	Humans and dogs

Campylobacter is transmitted between birds *via* the fecal-oral route, with only a small dose required (a concentration of around 35 cells) for successful colonisation (Cawthraw *et al*., 1996). This is because chickens are coprophagic animals, meaning they ingest excreted faeces. Therefore, faecal shedding is an important factor in the circulation of organisms among broiler flocks, once the first bird becomes colonised (Newell and Fearnley, 2003). Upon colonisation of one bird in a flock, almost all the birds (up to 100 %) will become colonised within a few days, due to rapid horizontal transfer (Coward *et al*., 2006) and after introducing chicks into a broiler house, most flocks will become infected 2 to 3 weeks later (Newell and Fearnley, 2003).

Campylobacter infection is acquired by humans through foodborne transmission, which is often linked to the consumption of undercooked broiler chicken, cross-contaminated foods, or untreated water. It is assumed, generally, that poultry meat is contaminated by campylobacters during processing, despite improvements in biosecurity to help maintain *Campylobacter*-negative flocks (Coward *et al*., 2006),

and that it survives through the food chain supply, becoming a risk to humans (Newell and Fearnley, 2003). Humans may also acquire *Campylobacter* through environment-to-human transmission due to contamination in agricultural environments and on farm equipment (Johnson, Shank and Johnson, 2017).

Researchers have also discovered the potential of *Campylobacter* transmission from broilers to humans through egg products; the moist membranes of eggshells allow them to survive in those environments (El-Saadony *et al*., 2023). This may occur through bacteria-containing waste which can stick to the eggshell. Moreover, Cox, *et al*., (2002), conducted a study that involved isolating *Campylobacter* spp. from the semen of broiler roosters. Results concluded that out of 275 semen samples, 10. % contained 'naturally occurring *Campylobacter'*, with counts as high as 1000 CFU ml^{-1}. Therefore, it has been suggested that rooster semen may act as a vector for the transmission of *Campylobacter* to the reproductive tract of the hen and then to the egg. Although it is reported that campylobacters are not detected in eggs (reviewed by Cox *et al*., 2012), another study (Hiett *et al*., 2002) discovered the presence of *Campylobacter* in the oviduct, which may result in its transmission to the egg at laying. This can also lead to the spreading of *Campylobacter* among the flocks as during hatching, the chicks may ingest bacteria and subsequently excrete it to colonise entire flocks (Cox *et al*., 2012).

1.5. *Campylobacter* epidemiology and symptoms in humans

Campylobacter has long been a major clinical and public health concern, emerging as a significant pathogen over the last three decades. Concerns have been present worldwide, with focuses on food products, such as poultry and water sources since 1977, when *Campylobacter* emerged as the major causative agent of acute diarrhoea (Igwaran and Okoh, 2019). *Campylobacter* has been the leading cause of gastrointestinal infection recorded in the last twenty years (Firlieyanti, Connerton and Connerton, 2016), in high-, middle- and low-income countries and it is responsible for 400-500 million diarrhoeal cases each year (Gahamanyi *et al*., 2020). It is estimated that 1.5 million people in the United States are affected by *Campylobacter* illnesses every year (The US Centers for Disease Control and

Prevention, 2024) and the Food Standards Agency estimates that there are 299,000 human foodborne cases of *Campylobacter* per year in the UK.

Ford *et al.,* (2023), carried out a study that examined the epidemiology of US *Campylobacter* infections from 2005 to 2018. Information received by The Foodborne Diseases Active Surveillance Network used laboratory-confirmed *Campylobacter* cases from 10 sites across the US. Over the examination period, 17066 isolates were received. There were 2449 records from 2017-2018, of which 88.4 % of the isolates were identified as *C. jejuni* and 11.6 % *C. coli*. The majority of cases were found among males, at 55 % and more than a third of cases, over 35 %, occurred during the summer period, similarly to *Campylobacter* prevalence in broilers. Over 98 % of patients experienced symptoms of diarrhoea, 32 % experienced bloody diarrhoea, 66 % reported fever, > 17% were hospitalised (< 45 % of these patients were hospitalised for 3 or more days) and 0.2 % of cases were fatal.

Zerbato *et al*., (2024), investigated human *Campylobacter* infections in Italy from 2017-2021 and collected data from 19 hospitals in 13 Italian regions. Since 2005, campylobacteriosis has been the highest reported zoonotic disease in Europe, according to the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC). In 2021, the ECDC revealed almost 128,000 cases of campylobacteriosis, which represented < 62 % of all zoonotic diseases reported in Europe that year. Zerbato *et al*., (2024) isolated 5419 *Campylobacter* spp. samples from faeces (~ 97 %) and blood (~ 3 %). Mirroring previous trends, the most frequently isolated species was *C. jejuni*, accounting for ~ 83.7 % of isolates, followed by *C. coli* at ~ 13.5 % and *C. fetus* at 0.6 %. *C. upsaliensis* and *C. hypointestinalis* were also isolated from a small number of cases. In addition, the majority of patients were males, at < 57 %; similar to the Ford *et al*., (2023) study. Moreover, the ECDC reported that higher infection rates were observed in males in Europe, in 2021, with a male to female ratio of 1.2:1. Another study in Italy, by García-Fernández *et al*., (2018), also indicates a statistically significant association between campylobacteriosis and gender, with a higher proportion in males. Furthermore, to the point of higher *Campylobacter* prevalence in broilers in the summer, Zerbato *et al*., (2024), recorded a ‘constant, reproducible seasonal trend’ in the incidence of campylobacteriosis among humans in the summer

months. An evident increase in prevalence was observed during these months, with lower rates of infection during winter and spring. Additionally, García-Fernández *et al*., (2018), reported that during 2013-2016, in Italy, 45 % of the yearly cases were reported from June to August.

Campylobacter infection is reported as endemic in African, Asian and Middle Eastern regions, and in these developing areas, infection is usually associated with children, with infection ratios being recorded to decrease with age. This has suggested that exposure in early life might lead to protective and population-level (herd) immunity. This term refers to the immune response of a host against an infection, either due to previous infection or vaccination, which can provide future protection against the disease. This immunity might reflect why asymptomatic *Campylobacter* infections are common in developing areas and this is suggested to have an impact on the transmission of these infections due to asymptomatic excretion. Furthermore, most *Campylobacter* shedders in these areas are asymptomatic, which causes difficulties with case recordings and epidemiology (Kaakoush *et al.,* 2015).

Gahamanyi *et al*., (2020), conducted a systematic review on the prevalence of *Campylobacter* species in humans and animals in Sub-Saharan Africa. Upon the screening of 33 articles surrounding human prevalence, Nigeria reported the highest, at almost 63 %, followed by Malawi at 21 % and South Africa at 20 %. The mean prevalence over all countries of all ages was 18.6 % and in children under 5, it was 9.4 %. The overall prevalence in humans in Sub-Saharan Africa ranged from 1.7 %-62.7 %. These figures further highlight the high prevalence of *Campylobacter* in humans, in countries all over the world but particularly in low- and middle-income countries, where campylobacteriosis is hyperendemic. This is likely due to poor sanitation, lack of knowledge, slow structural development and the close proximity of humans and animals.

According to the WHO (2020), the onset of symptoms of campylobacteriosis usually occurs 2 to 5 days after infection but it can range from 1 to 10 days. It causes diarrhoea (usually bloody), abdominal pain, fever, headaches, nausea and vomiting, with symptoms lasting from 3 to 6 days. Although most *Campylobacter* infections

are mild, self-limiting and are usually resolved within a week, without antibiotic intervention, extreme or extended infections can arise (Luangtongkum *et al*., 2009) that can be fatal in very young, elderly or immunocompromised patients. There have also been reports of complications such as hepatitis and pancreatitis and post-infection complications include reactive arthritis and Guillain-Barre syndrome; a form of paralysis that leads to respiratory issues and neurological dysfunction. Table 2 below outlines some diseases and infections that may be caused by certain *Campylobacter* species, in humans and in animals (Silva *et al*., 2018; Connerton and Connerton, 2017).

Table 2. *Campylobacter* species and related disease information.

Species	**Disease information**
Campylobacter coli	Can cause gastroenteritis in humans and animals, meningitis, bacteraemia and sepsis in humans and hepatitis in animals.
Campylobacter concisus	Can cause gastroenteritis, IBD, oesophagus complications, gum disease, brain abscesses and reactive arthritis in humans
Campylobacter curvus	Can cause gastroenteritis, ulcerative colitis, oesophagus complications, gum disease, and bronchial and liver abscesses in humans
Campylobacter fetus subsp. *fetus*	Can cause gastroenteritis, septicaemia, meningitis, endocarditis and brain abscesses in humans and abortions in humans and animals
Campylobacter jejuni subsp. *doylei*	Can cause gastroenteritis and septicaemia in humans
Campylobacter jejuni subsp. *jejuni*	Can cause IBD, reactive arthritis, Guillain-Barre syndrome, septicaemia and meningitis in humans and abortion and gastroenteritis in humans and animals
Campylobacter lari subsp. *lari*	Can cause septicaemia in humans and gastroenteritis in humans and animals
Campylobacter rectus	Can cause gastroenteritis, IBD, gum disease, thoracic empyema and vertebral abscesses in humans

A WHO and Food and Agriculture Organisation risk assessment of *Campylobacter* spp. presence in broilers concluded that there is a linear relationship between flock prevalence and the probability of human campylobacteriosis (Risk assessment of

Campylobacter spp. in broiler chickens. Technical report. Microbiological Risk Assessment Series (MRA) 12 (who.int)). Therefore, the need to reduce flock prevalence is pivotal as this will substantially reduce the cases of human disease (Newel and Fearnley, 2003).

1.6. *Campylobacter* prevalence, persistence and symptoms in broiler chickens

Campylobacter jejuni has been seen to colonise the colon of broiler chickens to very high numbers, up to 10^9 CFU per gram of caecal contents (Coward *et al*., 2006). It also colonises additional organs such as the small intestine, liver and spleen; *C. jejuni* can cross the intestinal epithelial barrier and ease the translocation of *Campylobacter* spp. and other enteric organisms such as *E. coli* to organs outside of the intestines (Awad *et al*., 2016). Estimates of *Campylobacter* contamination found on raw chicken sold in the UK has ranged from 40-80 % (Coward *et al*., 2006). However, the prevalence of *Campylobacter* in broiler farms varies depending on the age of the animals. Under conventional barn rearing systems, campylobacters are minimally detected in chicks aged 1 to 3 weeks and if infected, *Campylobacter* will spread throughout the rest of the farm, residing until slaughter and polluting the meat being processed (El-Saadony *et al*., 2023). The detection and dissemination of *Campylobacter* in faecal samples of colonised birds has proven quite frequent, due to the levels present in the gut and their coprophagic nature (Newell and Fearnley, 2003), which is consistent with the spread within flocks once one bird is colonised.

Investigations into the epidemiology of commercial broiler flocks have indicated that natural flock colonisation is dependent on the age of the birds (Newell and Fearnley, 2003). Newly hatched chicks in European flocks were found to be negative for campylobacters, which persisted until 10 days of age, when the lag phase of bacterial growth is said to be detected. This is not withstanding low transmission rates upon hatch noted above. The period of colonisation and shedding among these flocks is not fully known but it is generally said that colonisation lasts for the lifetime of the chicken, which is usually no longer than 47 days, for traditionally reared birds. However, in broilers reared and challenged for experiments, colonisation may persist in a varied manner among different *Campylobacter* strains, although, after 8 weeks,

the *Campylobacter* count recovered in caecal contents and the number of colonised birds may reduce.

Results from several studies have indicated that the proportion of broiler flocks colonised by *Campylobacter* varies greatly among different countries, aside from the potential differences from isolation and sampling methods used in these experiments (Newell and Fearnley, 2003). A yearlong study conducted in the US consisted of 32 broiler flocks on eight farms from multiple producers (Stern *et al*., 2001). Results determined that nearly 90% of the flocks were colonised by campylobacters and in the majority of these flocks, it was only detected in faecal and caecal samples once the chicks reached 4 to 8 weeks old. Other studies in Europe discovered values ranging from 18 to > 90 % of *Campylobacter* prevalence among broiler flocks, with the UK and Italy having the highest values of > 90 % and 80%, respectively. The countries that showed much lower prevalence values are in the Northern part of Europe, such as Sweden and Norway, which may be a reason for the low *Campylobacter* counts. This may be due to the climate, the distance between farms and the number of animals per farm. In addition, these farms may be better regulated and possess newer facilities than in other countries (Newell and Fearnley, 2003). Newell and Fearnly (2003), also suggest that flock positivity is dependent on the size and the nature of the production system. For example, positivity has been generally higher, up to 100 %, in organic and free-range flocks compared to those in intensive farming systems. This may be a result of environmental exposure, the size of the flocks and the age of the chickens at slaughter. Where species identification of the positive broiler flocks occurred, *C. jejuni* was observed to have colonised the majority; 80 to 90 %, with *C. coli* colonising the majority of the remaining flocks and *C. lari* also being detected.

In the study by Gahamanyi *et al*., (2020), 11/34 articles contained data on *Campylobacter* prevalence in chickens from five different countries in Sub-Saharan Africa; this was the highest number of articles on any reservoir. The mean prevalence was almost 63 %, again the highest figure among all animal reservoirs, with *C. jejuni* accounting for 81 % of cases and *C. coli* at just above 18%. Ethiopia reported the highest prevalence, at almost 87 %. The mean prevalence concluded in this review is similar to that found in Asian countries such as Thailand and India.

The study conducted by Jacobs-Reitsma *et al*., (2009), on Dutch farms, involved obtaining samples from hatchery and fresh litter, with the inclusion of water, feed, insects and domestic animal faeces present on the farms. The results identified that broiler flocks generally became colonised with *Campylobacter* at 3-4 weeks old, offering isolation figures of 100 %, and these birds remained colonised until slaughter. Infection by *C. jejuni* among broilers at slaughter has been recorded as high as 100 %, highlighting their high prevalence among commercial flocks.

In this study by Hald *et al*., (2004), strict biosecurity measures were put in place but failed to control the incidence of infection; in August 2003, in Denmark, where 72.1 % of flocks were infected and the sale of *Campylobacter*-contaminated chicken meat unavoidable. These numbers may be explained by the higher presence of flies in the summer, as they can act as vectors in the transmission of bacteria, especially through ventilation systems.

The infection of broiler chickens by *Campylobacter* spp. is so frequent as they employ a poor or inefficient immune response, so are tolerant to the organism. This causes caecal colonisation by *Campylobacter* to be persistent as it is not attacked readily by host immune responses (Konkel *et al*., 2020). Newell and Fearnly (2003), also state that colonised chickens fail to show clinical symptoms of infection, even when the chicks are exposed to high numbers of the organisms for experimental purposes. As broilers are frequently exposed to *Campylobacter* spp., at such young ages, Newell and Fearnly (2003) suggest that the lag phase of bacterial growth is likely to be an innate characteristic of the chick. In addition, during the first few weeks of life, the avian intestine undergoes several physiological changes such as the development of mucosal immunity and changes in the microbiota, which have an impact on the persistence of certain organisms. Changes in feed composition may also impact this. These factors may explain the increase in *Campylobacter* counts observed at 3-4 weeks of age.

However, it has also been reported that certain strains can cause a negative effect on chicken health and welfare but the symptoms of *C. jejuni* in poultry are not evident (Humphrey *et al*., 2014). Four commercial breeds of broilers were subjected to experimental infection and findings suggested that breed has a significant effect on

infection by *C. jejuni* and the subsequent immune response of the host. All breeds displayed an immune response. Some breeds remained healthy after a regulated intestinal inflammatory response was initiated upon expression of interleukin-10. However, another breed experienced damage to the gut mucosa and diarrhoea after a prolonged inflammatory response was initiated; these findings challenge the theory that *C. jejuni* is harmless to chickens. Nevertheless, it is still necessary to eliminate this organism in poultry to reduce the incidence of human infections.

1.7. Antimicrobial resistance (AMR) in *Campylobacter* spp.

AMR has been a major worldwide concern since the mid 20th century and has developed to become one of the most serious global public health and development threats. According to the WHO (2023), it was estimated that bacterial AMR was directly responsible for around 1.3 million deaths across the globe in 2019 and contributed to a further 4.95 million deaths. Over the past few decades, it seems that bacteria causing common or severe infections and diseases have developed resistance to each new antibiotic coming to market (Prestinaci *et al*., 2015). Humans are greatly responsible for the emergence of various antibiotic-resistant bacteria due to random misuse and overuse of antibiotics during the treatment of infections in clinical and agricultural settings.

According to Portes *et al*., (2023), who focussed on antibiotic-resistant *Campylobacter* in South America, *Campylobacter* has become increasingly resistant to antibiotics such as tetracycline, nalidixic acid and ciprofloxacin but especially the primary drugs used to treat campylobacteriosis. These primary drugs include Azithromycin and fluoroquinolones (CDC, 2024). Portes *et al*., (2023), states that the main sources of antibiotic-resistant *Campylobacter* were farm animals and foods from animal sources, from which they concluded that the resistant isolates were spread from multiple sources linked to animal husbandry in South America. The high level of resistance was thought to compromise the treatment methods of campylobacteriosis in humans and animals, a problem that quickly needs to be solved. Not only has antibiotic-resistant *Campylobacter* been identified in South America, but according to Luangtongkum (2009), several studies reported a rapid increase in the proportion of antimicrobial-resistant *Campylobacter* strains in many

other countries, compared to that from before this century. Before 1992, fluoroquinolone-resistant *Campylobacter* was rarely recorded in the USA and Canada but after this period, results indicated that 19-47 % of human-isolated strains were resistant to ciprofloxacin. Likewise, in Europe, 17-99 % of *Campylobacter* strains isolated from humans and animals were recorded to be fluoroquinolone resistant.

The study by Ford *et al*., (2023), recorded high levels of resistance by *Campylobacter* to several human antibiotics, during 2017-2018. Tetracycline experienced the highest resistance levels at ~ 45 %, followed by ciprofloxacin at just under 30 %. Campylobacters are also resistant to Clindamycin, Telithromycin, Erythromycin, Gentamicin and Florfenicol but at much lower incidences. Ciprofloxacin experienced a < 5 % increase in resistance in 2017-2018 compared to the previous decade; resistance was also increased for several other antibiotics.

There have been several attempts by different countries/ groups of countries to monitor the incidence of *Campylobacter* in food products, especially meat, where the USA, EU member states and Australia/New Zealand are the most advanced in the deployment of regulatory frameworks (Portes *et al*., 2023). For example, the USDA released a range of maximum acceptable percentages of positive samples for various meat sources. In addition, the EU released a risk assessment framework and model for *Campylobacter* in broiler chickens, outlining an acceptable maximum quantity of *Campylobacter* found on carcass samples after refrigeration. However, alternative methods to treating the infection remain of high importance to enable compliance.

The WHO (2017) published a list of antibiotic-resistant ‘priority pathogens’ that pose the greatest threat to human health. The aim was to encourage and promote the research and development of new antibiotics to address the increasing global resistance of bacteria to antimicrobial treatments. The major threat lies with gram-negative bacteria that are multidrug-resistant (MDR). MDR bacteria have naturally developed the ability to resist treatment through various mechanisms and transfer this ability to other bacteria through their genetic material. As *Campylobacter* is resistant to various antibiotics including fluoroquinolones and macrolides, the most widely used antimicrobials for clinical campylobacteriosis treatment, the WHO labelled it as

being of ‘high’ priority, in second place to those bacteria of ‘critical’ priority, requiring alternative treatments (WHO, 2017).

The widespread use of antibiotics against bacteria such as *Campylobacter*, has warranted the urgent demand for alternative treatment methods due to the constant, increasing emergence of resistant strains across the globe, posing a major threat to public health. This is particularly important as there is a consequential increase in immunosuppressed patients who may experience fatal side effects of infections. Although antibiotic resistance, from *Campylobacter* infections, is a major issue among humans, when treating *Campylobacter* in broilers, there is no antibiotic treatment available, so alternative antimicrobial agents, such as bacteriophages, are under consideration.

1.8. Bacteriophages and phage therapy

1.8.1. Background

Bacteriophages (phages) are viruses that infect and replicate in bacteria and archaea. They are the most abundant organisms on the planet and are therefore, ubiquitous in the environment (Clokie *et al*., 2011), highlighting their accessibility for disease treatment and biocontrol. Phages are very diverse in their size, genomic orientation, morphology and host ranges and they are very species-specific when infecting bacteria and sometimes even strain specific. Phages were first discovered in 1915 by William Twort, according to Aswani and Shukla (2021), which led to the early attempts of using bacteriophages to treat infections worldwide. The first clinical application was conducted in Paris, in 1919, for the treatment of dysentery, and from 1920 to 1940, there was an increase in the use of phages to treat diseases. Phage efficacy for clinical use is determined using several factors, including how greatly phages can reduce bacterial populations, the diversity of host ranges and efficiency of plating (EOP), which determines how many target cells can be infected by the phage.

1.8.2. Bacteriophage structure and taxonomy

The structure of bacteriophages is well-suited to their function within mixed communities of microorgansims; using their structures to bind and infect specific host bacteria. Phages are composed of genetic material, either double or single stranded (ds or ss) DNA or RNA, enclosed in a protein capsid. There are three basic structural forms: an icosahedral head with a tail, an icosahedral head without a tail and a filamentous form. These forms have served as a basis for taxonomy but recently these have been set to one side in favour of molecular genetic information (Turner *et al*., 2023). The majority of *Campylobacter*-specific phages are lytic with myoviral morphologies that are classified in the Eucampyvirinae subfamily. The phages are nonenveloped with a long, contractile tail and an icosahedral head, that contains AT-rich- and dsDNA. *Campylobacter* phages are further divided into one of two genera: Fletchervirus and Firehammervirus, based on their DNA sequence; Firehammerviruses are group II phages (Figure 2A) and Fletcherviruses are group III (Figure 2B) phages (Javed *et al*., 2013).

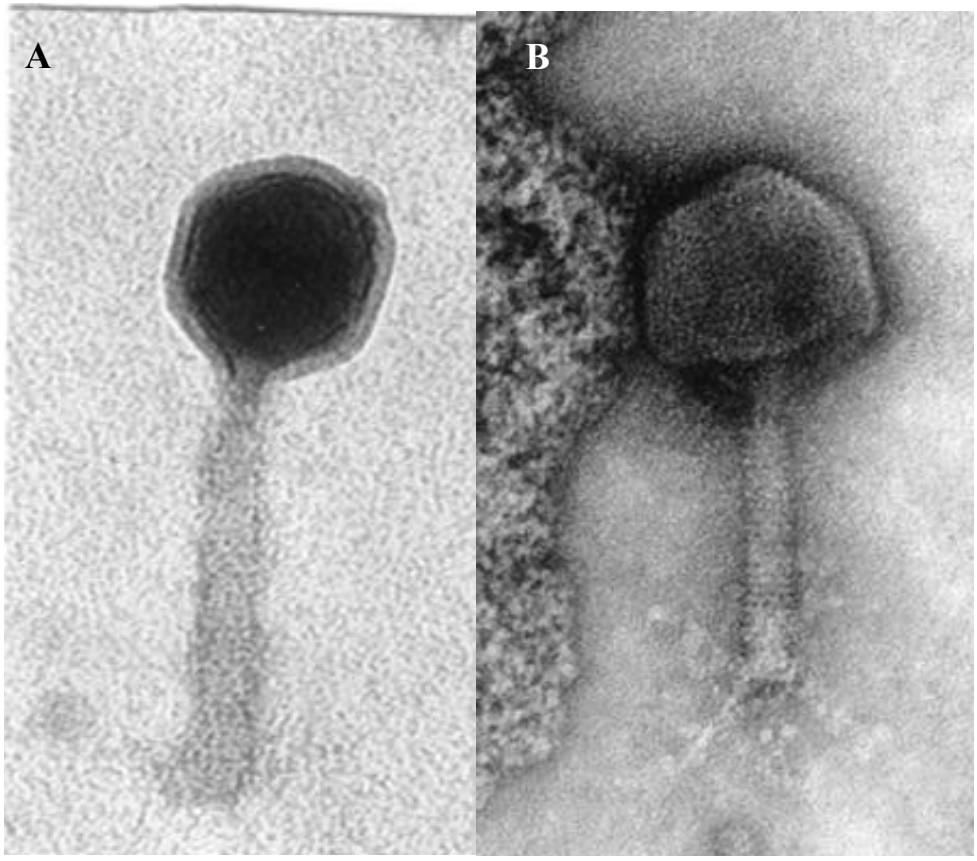

Figure 2. Electron micrograph of *Campylobacter* phages. (A) Firehammervirus, group II, type CP220. (B) Fletchervirus, group III, type CP81. Phages have icosahedral heads with tail tubes and sheaths and short, thin tail fibres. Group II phages have longer, extended tail tubes, compared to those in group III (Javed *et al*., 2013).

Group II phages generally have a smaller protein head diameter of 83 to 99 nm, compared to those in group III, of 100-130 nm (Olson *et al*., 2022). The genome size is ~ 184 kb in Firehammerviruses and ~ 138 kb in Fletcherviruses (all virulent *Campylobacter* phages are divided into three groups, based on their genome size). Group I phages are larger, with genomes reported at 320 kb and head diameters of 143 nm, group II phages infect *C. jejuni* and *C. coli*, through recognition via the flagellum, whereas group III phages generally infect *C. jejuni* and bind to their host via the capsular polysaccharides (Steffan *et al*., 2022).

1.8.3. Replication and survival strategies

Phages are obligate parasites of bacteria so they rely fully on their host's extracellular and intracellular machinery to replicate (Holtappels *et al*., 2023). Phages use 1 of 2 replication strategies upon infection of their host; undergoing either the lytic or the lysogenic life cycle (Kasman and Porter, 2022). The lytic (virulent) life cycle (Figure 3) involves killing host cells by injecting the phage genome into the cytoplasm, where the virus then replicates its genome and shuts down bacterial protein, DNA and RNA synthesis. This genome replication then forms new viruses using the host cell manufacture, which then burst out of the cell to infect more host cells (Kaiser, 2016). The lysogenic (dormant) life cycle (Figure 4) is less common and phages that infect hosts using this method are known as temperate phages. This cycle involves the injection of the virus genome into the host cell, which is then integrated with the host DNA, by recombination, to replicate alongside it and become a prophage. The virus stays dormant until the host cell comes under stress, then the phage become active and initiate reproduction, resulting in lysis of the host cell. Lysogeny allows the host cell to maintain survival and reproduce and due to the integration of DNAs, the virus is reproduced in the bacterial offspring (Kaiser, 2016).

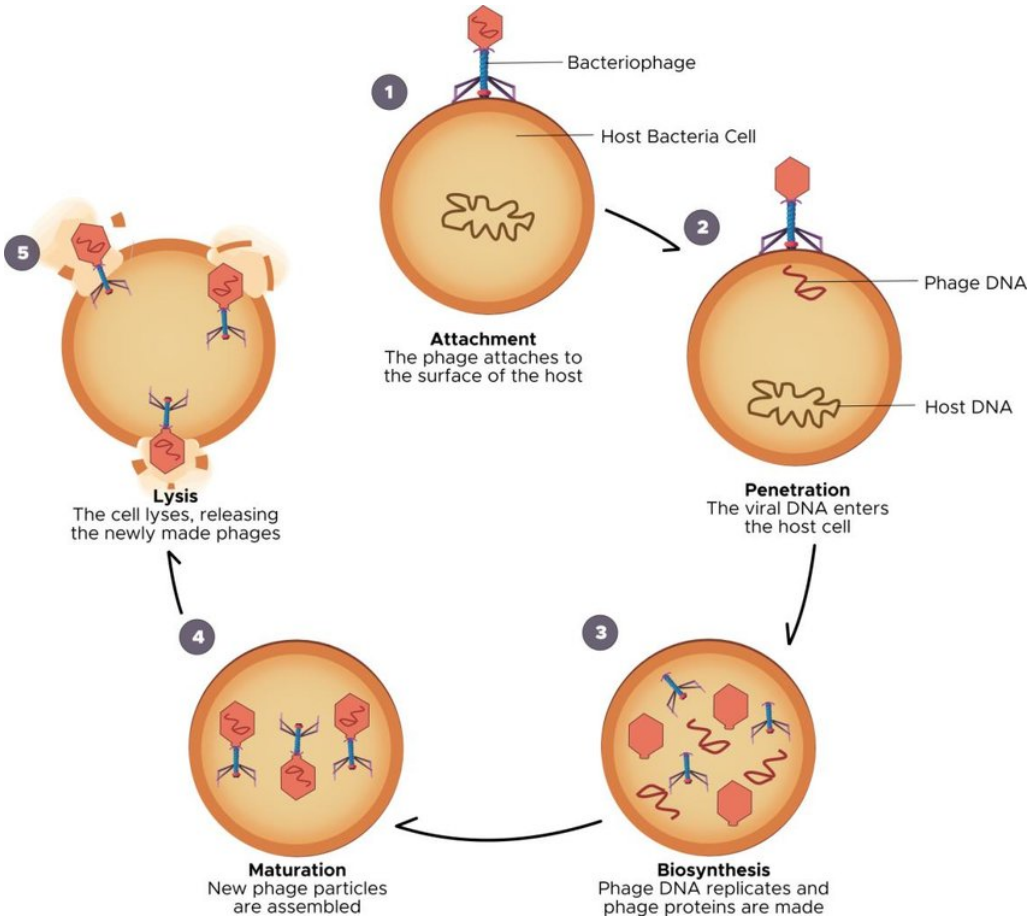

Figure 3. The lytic (virulent) lifecycle of bacteriophages (Adesanya *et al*., 2020)

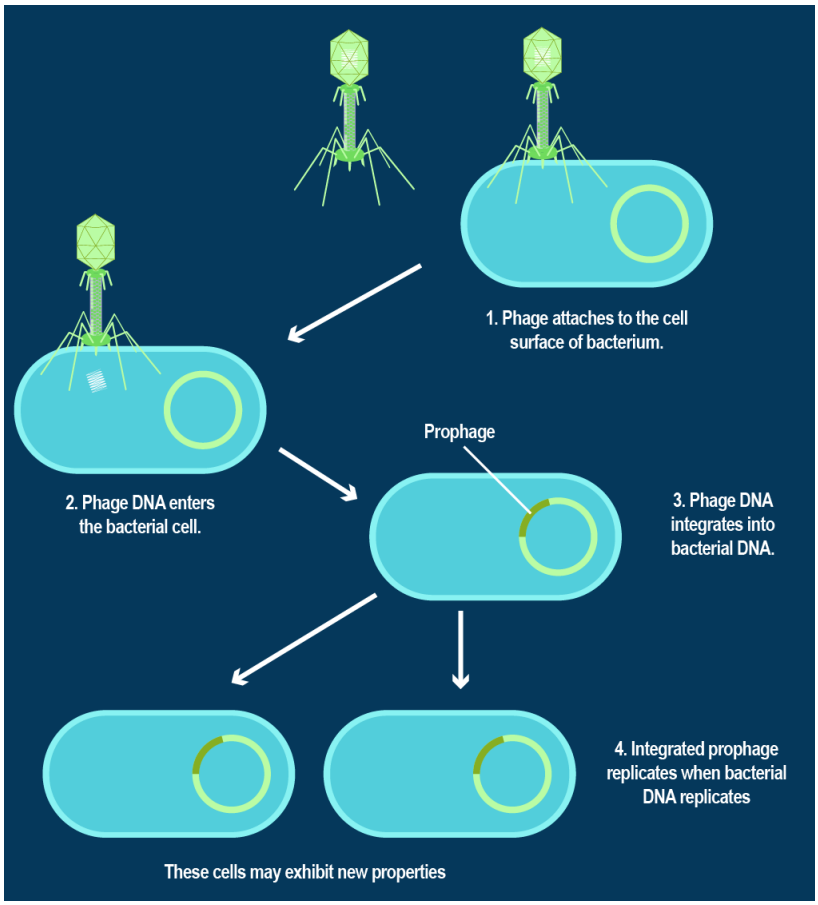

Figure 4. The lysogenic (dormant) lifecycle of bacteriophages.

Op. cit: Steward, K. (2018). *Lytic vs Lysogenic – Understanding Bacteriophage Life Cycles*. [online] Technology Networks, Immunology & Microbiology. Available at: https://www.technologynetworks.com/immunology/articles/lytic-vs-lysogenic-understanding-bacteriophage-life-cycles-308094.

As they are members of the *Myoviridae* family, *Campylobacter*-specific phages usually employ the lytic life cycle upon infection of their host. However, in some cases, the phages enter the carrier state life cycle to maintain the phage population in association with its host, in equilibrium (Hooton *et al*., 2016). This is a complex relationship, which offers phages shelter and continued replication while they translocate between environments abundant of their host bacteria, such as animal intestinal tracts. It also offers advantages to the host bacteria, allowing them to survive in environments outside of the intestine. The difference between the lysogenic and carrier state life cycles is such that in the lysogenic life cycle, the virus genome is injected into the host cell and is integrated within the host DNA so both

DNAs replicate together. The carrier state life cycle involves mixtures of bacteria and phages which survive alongside each other in a stable environment. Furthermore, some of the target bacteria are resistant to the carrier state phage, evading infection, while the sensitive variants maintain the phage population so that both the bacteria and phage thrive (Siringan *et al*., 2014).

1.8.4. Bacteriophage therapy in clinical settings

Studies and reports surrounding phage activity and phage therapy have featured in scientific literature since shortly after their discovery. However, with the rise in antibiotic resistance, the practise of phage therapy as an antimicrobial is becoming increasingly more frequent. In many cases, the success of bacteriophage therapy, in the treatment of patients with infectious diseases, has been effective but has required careful management of the case selection and application. The overall success of phage therapy is dependent on several factors: phage efficacy, which coincides with how well the patients have recovered, and how safe the therapy is, whether there are adverse side effects after application. The efficacy of phages is also dependent on several elements such as the host range (the range of bacteria it can infect, at the genus, species or strain level), lytic activity (how well the phage can infect bacterial cells), dosage (how much is needed for successful treatment, but the phages themselves can establish the dose after application), route (where the phages are administrated in patients), how ubiquitous the particular type is in the environment, the duration of administration, the level of bacterial load and the ability of the target bacteria to develop resistance to the phage (Fang *et al*., 2024).

In an article from The Lancet Infectious Diseases journal (Uyttebroek *et al*., 2022), published clinical data was evaluated to demonstrate the safety and efficacy of phage therapy. Fifty-nine studies between 2000 and 2021, involving over 2200 patients, treated with phage therapy, offered positive results. The safety of phage therapy was reported in a large majority of the studies and explained that the high specificity of the phages for their host bacteria, avoided harm towards the commensal microbiota so the incidence of adverse effects was very low. In addition, all studies outlined the efficacy of phage therapy; health care improvement was seen in 79% of patients and in 87%, bacterial eradication was reported.

A report published in Poland (Weber-Dąbrowska *et al*., 2001a), records the efficacy of phage therapy as a treatment method for 1307 patients that suffered from suppurative bacterial infections, caused by multidrug-resistant bacteria of different species. It was recorded that the therapy was highly effective, with a full recovery seen in almost 86% of patients and temporary improvements seen in almost 11%. Phage therapy was used on these patients as their infections were failing to respond to the available antibiotic treatment and these results confirmed the high efficacy of phage therapy, particularly against multidrug-resistant bacteria.

A further study (Weber-Dabrowska *et al*., 2001b) investigated the effectiveness of bacteriophage therapy against infections in twenty cancer patients, aged 1-66, as they are known to be immunocompromised and susceptible to infections. The patients had simultaneous infections with a range of different bacteria including *S. aureus* and *E. coli* which had been treated previously with antibiotics but experienced no response. Bacteriophages were matched specifically to the isolates found in the patients and were used for treatment as the infectious bacteria were resistant to antibiotics. The outcome was very positive; infections were cured in all patients, which indicated a high efficacy from the phage therapy approach, particularly as antibiotic therapy had failed. In addition, results further indicated that bacteriophage therapy could upregulate immune responses of cancer patients.

In therapeutics, phages are often used in combination with other phages, to create phage cocktails. These aim to target a range of bacteria, improving the breadth of activity and they also reduce the likelihood of bacteria evolving phage resistance (Abedon *et al*., 2021). Yoo *et al*., (2023), published a study that designed phage cocktails to target phage-resistant mutants of *Klebsiella pneumoniae*, which were also multi drug-resistant (MDR). Four phages were isolated that targeted different bacterial surface molecules and three phage cocktails were constructed. The different phage cocktails were also tested for their ability to suppress adsorption-blocking resistance among the bacteria. The resensitisation phenomenon was a focus point and outlined: bacteria that had acquired resistance to one phage became susceptible to those phages if they acquired resistance to different phages. This phenomenon offers the potential to inhibit the emergence of phage-resistant bacteria. Phage resistance also resulted in decreased growth rates and reduced virulence among the bacteria due

to the trade-off phenomenon. Yoo *et al*., (2023) concluded that if one of the cocktails from this study was used for the treatment of MDR bacteria, direct bacterial lysis would occur, along with indirect therapeutic effects.

Based on the outcomes of the above reports, data suggests that phage therapy can provide successful clinical results in patients with a variety of different bacterial infectious/diseases, which failed to respond to previous antibiotic treatment (Międzybrodzki *et al*., 2012). The success of phage therapy has been seen in immunosuppressed cancer patients with bacterial infections, patients with suppurative bacterial infections, those with nonhealing ulcers, infected burns and in many more cases. It is further suggested by Weber-Dabrowska *et al*., (2001b), that bacteriophage therapy is the only successful and safe treatment available against infections caused by *Staphylococcus*, *Escherichia*, *Klebsiella*, *Enterobacter*, *Proteus* and *Pseudomonas*, due to their ability to resist antibiotics. These previously recorded successes against human infections offered a positive prospect for phage application in broiler chickens, to eradicate the increasing issue of *Campylobacter* infections acquired from poultry.

1.8.5. Bacteriophage therapy in livestock production

As stated previously, the extensive use of antibiotics is not only a problem in clinical settings, but more so in agriculture; livestock farming is responsible for 50-80 % of total antibiotic use in developed countries. This includes using antibiotics in animal feeds for pigs, poultry and cattle. Not only have antibiotics been used for treating bacterial infections in livestock, but they have also been commonly used as growth-promoting factors since the mid 1900s, further contributing to antimicrobial resistance. As a result of this, the banning of antibiotics for growth promotion was widely observed in Europe after the mid 1990s and the discovery of bacteriophage alternatives was encouraged (Ferriol-González and Domingo-Calap, 2021).

Ferriol-González and Domingo-Calap (2021), reported that bacteriophage therapy for the treatment of infections affecting cattle and pigs, has been successfully tested. Ngassam-Tchamba *et al*., (2020), investigated the efficacy of three lytic bacteriophages against *Staphylococcus aureus*, isolated from cows infected with mastitis in Belgium and Norway. They were assessed *in vitro* and *in vivo* using

mouse and *Galleria mellonella* models. Results showed that only 50 % of larvae in *G. mellonella* survived within four days of *in vivo* treatment with the three phages. Results in the mouse models were not as successful.

Studies have also focussed on the control of zoonotic diseases from farm animals (Zia and Alkheraije, 2023). Gigante and Atterbury (2019), reported several investigations into the use of phages to control zoonotic pathogens such as *Salmonella* in intensively reared livestock, including pigs. One investigation consisted of administering 5 x 10^9 CFU of *S. typhimurium*, along with 5 x 10^9 PFU of a phage cocktail (which consisted of sixteen different phages) to piglets. A 2 to 3 $\log_{10}$ CFU g^{-1} reduction in *S. typhimurium* counts was recorded in the ileum, caecum and tonsils of the pigs (Wall *et al*., 2010).

These studies highlight successes of bacteriophage therapy in livestock production (Bianchessi *et al*., 2024). Although there are applications that have proved challenging, there are many others that report positive results against other pathogens such as *E. coli*., *Clostridium* spp., *Listeria* spp., and further *Salmonella* spp. Therefore, this should encourage agricultural workers to avoid using antibiotics by any means in this industry, to prevent the further emergence of MDR bacteria, and move towards using bacteriophages.

1.8.6. Advantages and disadvantages of phage therapy

The advantages of phage therapy greatly outweigh the disadvantages, which is a promising prospect. Loc-Carrillo and Abedon (2011), published a study outlining the pros and cons of phage therapy and the properties of phages that may contribute to their clinical utility, these are outlined below.

1.8.6.1. Advantages

Phages are bactericidal. Cells that have been successfully infected by lytic phages are no longer viable. This offers more success compared to some antibiotics, which may promote evolution to resistance among bacteria.

Phages can be applied in a single dose, which exploits the ability of phages to replicate rapidly. Upon single dose application, they carry out ‘automated dosing’. During the infection of hosts, phages replicate in the lytic cycle (Figure 3), where they increase their population substantially, in the host environment, establishing the phage dose, to infect as many cells as possible. However, they can only do this if the host population is high enough and single dosing should not be expected in every instance, to be sufficient for treatment. If a single dose may be insufficient, many phages may require only low doses, which could potentially be more economically efficient. When considering the cost efficiency of phage application, phage production is no more expensive than pharmaceutical production and phage isolation procedures and characterisation is not economically demanding.

Phages are safe to use. They are mostly composed of nucleic acids and proteins so are naturally harmless. However, in some instances, phages can interact with the immune system which could result in harmful responses, but this is unlikely to be a concern during treatment. As a result of this, certain phage procedures may use highly purified phage preparations.

The normal microbiota in the human gut is only minimally disrupted by phages. Due to their host specificity, targeting only a few bacterial species or sometimes more than one closely related genus, they can protect the normal microbiota bacteria. On the other hand, antibiotics can cause superinfections, such as antibiotic-associated *Clostridium difficile* colitis. Furthermore, their host specificity restricts the number of bacteria that can develop phage-resistance mechanisms.

The mechanism of infection by phage is different from those employed by antibiotics. Therefore, phages can kill bacteria that have developed antibiotic resistance mechanisms such as *Staphylococcus aureus*, which is MDR. In addition, phages have the ability to clear some biofilms, more so than antibiotics, as they can penetrate through them, lysing bacterial layers individually. Biofilms are commonly resistant to antibiotics due to complexity in their structure, which is made up of exopolysaccharide, DNA and proteins (Prinzi and Rohde, 2023). Furthermore, the utility of phages does not contribute to antibiotic resistance among bacteria so using

them to replace antibiotics could improve the AMR issue and extend the clinical use of antibiotics.

Bacteriophages are ubiquitous and natural in the environment. Phages are the oldest and most abundant organisms on the planet; there is an estimated 10^{31} bacteriophage particles in the biosphere (Strathdee *et al*., 2023). This allows for their easy discovery, mainly in sewage and other waste materials, where there are high numbers of bacteria. In addition, phages that present little or no toxicity can be isolated through cultures of most target bacteria.

Phages are very versatile. They can be used in combination with antibiotics, probiotics, alternative antimicrobials, surfactants and vaccines. They can also be used in combination with other phages (phage cocktails), which broaden their host ranges and enhance their bacterial infection ability. Phages are also versatile in the application sense; they can be used in liquid form, in creams and solids as well as having multiple administration routes.

Further studies enable evaluation of the effect of each measure as combined counter measures to discover the most favourable approach to the treatment of bacterial infections (Fang *et al*., 2024).

1.8.6.2. Disadvantages

The high specificity of phages and narrow host ranges means they can only infect certain bacterial genera and are sometimes limited to few species and strains. Therefore, for infections caused by various harmful bacteria, it may be difficult to successfully treat them using bacteriophages that are specific to only certain species. However, there has been extensive use of phage cocktails in therapeutics which target more strains of bacteria and reduce the likelihood of evolving phage-resistant bacteria (Abedon, *et al*., 2021).

Phages that employ the lysogenic life cycle (temperate phages) when infecting bacteria inhibit the lytic ability of other phages when they try to infect the same bacteria, their host. These lysogenic phages can also transmit toxins and antibiotic

resistance genes, or enable phage resistance, to the bacteria upon infection. However, in clinical settings, temperate phages are rarely used so this fails to be a frequent challenge; not all phages would prove successful in therapeutics. There are some phages that possess inefficient infection mechanisms such as poor adsorption strategies, inability to avoid bacterial defence mechanisms and poor replication strategies. Phages need to be efficient at reaching and infecting bacteria without negatively impacting their environment. This can be achieved with strictly lytic phages if they are stable under usual storage conditions (Lin *et al*., 2022).

There are emerging bacteriophage-resistant strains of bacteria. This happens if a single phage is used repeatedly for a long period of time but these strains can also evolve through natural selection. They can evolve several phage-resistance mechanisms such as injection blocking, adsorption blocking, immunity of superinfection and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs). Inhibiting adsorption reduces the level of interaction between bacteriophage and bacteria. The CRISPR-Cas system is part of the adaptive immune system and can provide immunity to bacteria against bacteriophages by interfering with foreign invaders. They achieve this through gathering CRISPR and Cas proteins together that target and cleave invasive nucleic acids. At the genome level, the CRISPR-Cas system can add or delete gaps in host genomes or induce mutations or deletions in phage genomes (Lin *et al*., 2022). CRISPR spacers that contain bacteriophage sequences have been identified in some *C. jejuni* strains which could present a challenge in poultry farming as they can confer resistance to phage treatment. However, the majority of the CRISPR spaces identified have been directed at temperate phages (Yeh *et al*., 2024).

There is considerable unfamiliarity in the Western world surrounding the knowledge of phages as antimicrobial agents which presents a challenge due to the scepticism around their clinical use. However, the range of advantages prove that they are a favourable method in the treatment of bacterial infections, encouraging Western medicine to comprehend their full potential. Overall, phage application is cost-effective, safe and easily applied by farmers so proves an important practice in public health, through reducing the incidence of bacterial infection.

1.8.7. Bacteriophage therapy in broiler chickens

Colonisation of broilers by *Campylobacter* spp. is pervasive and difficult to inhibit and bacteriophages are a promising measure in the biocontrol of this pathogen. *Campylobacter* bacteriophages are natural predators of *Campylobacter* spp. such as *C. jejuni* and *C. coli* and have been repeatedly reported to reduce *Campylobacter* counts in poultry products. There are few, if any, current alternatives for *Campylobacter* treatment, as antibiotic use is inappropriate and potentially dangerous and there is an absence of vaccines available for commercial use. There have been several reports published worldwide, studying the efficacy of different bacteriophage treatments in reducing the colonisation of *Campylobacter*, mainly *C. jejuni*, in broilers. Furthermore, studies on the presence of bacteriophages in chicken livers have also been published, including that by Blanco-Picazo *et al*., (2022), which concluded that phages can translocate from the gut to the liver.

Loc-Carillo *et al*., (2005), published findings surrounding the efficacy of *Campylobacter* phages in reducing *Campylobacter* counts in experimentally infected broilers. Low-passage *C. jejuni* isolates HPC5 and GIIC8 were obtained from UK broiler flocks and used to colonise the experimental models. Two phages: CP8 and CP34 were identified as having a broad host range and were subsequently orally administered to the experimentally colonised broilers. *Campylobacter* counts in these birds reduced by 0.5-5 $\log_{10}$ CFU g^{-1} of caecal contents, compared to untreated control birds, depending on the phage-*Campylobacter* combination, the dose of phage and the time post administration. The treatment of *C. jejuni* GIIC8 colonised birds with phage CP8 resulted in the greatest reduction in *Campylobacter* counts, of up to $\log_{10}$ 5.6 CFU g^{-1} within 24 h of administration. Overall, phage CP34 was more effective in reducing *C. jejuni* counts at all sites; upper intestine, caeca and lower intestine, compared to CP8. Bacteriophage-resistant Campylobacters were present but were only isolated at a frequency of < 4% from phage-treated chickens and their colonisation ability was compromised. Overall, this data showed that these bacteriophages possess the characteristics needed to control *Campylobacter* counts in broilers and that this is a sustainable biological control measure for *Campylobacter* reduction in these birds.

A further study, by Chinivasagam *et al*., (2020) described the development and utility of bacteriophage cocktails to control *Campylobacter* counts among broilers on farms in Queensland, Australia. The monophages in the cocktails were selected based on their efficacy against the maximum number of *C. jejuni* and *C. coli* isolates on the farms and *Campylobacter* counts were assessed after rearing, after transport and post slaughter. Reductions in *Campylobacter* counts were observed in the caeca on Farm A, approx. 28 h post phage treatment; these ranged from 1-3 $\log_{10}$ CFU g^{-1} compared to controls. On Farm B, there was no significant difference in the caecal counts between the treatment and control groups post treatment but a reduction of 1.7 $\log_{10}$ CFU g^{-1} was observed from treated birds collected the week before. Campylobacters isolated from both farms were sensitive to the treatment phages and the study indicated that bacteriophages isolated from these farms have potential to control intestinal *Campylobacter* counts in broilers in this part of the world. In addition, no resistance to the phage cocktails on either farm was detected among the isolates. 24 h was given between treatment and slaughter, which minimised the time for phage-resistant strains to emerge but on the other hand, may have decreased the effectiveness of treatment compared to results that might have been seen after 2-4 days post treatment and pre slaughter.

Richards *et al*., (2019), conducted a further study on the biocontrol of *C. jejuni* in chickens, by phage, while also proving that there were no negative impacts on the gut microbiota. This finding was necessary for phage therapy in understanding the possible effects of the treatment on the chickens, to construct a sustainable practice. A cocktail comprised of two virulent *Campylobacter* phages: CP20 and CP30A, was used to treat broilers that had been colonised with *C. jejuni* HPC5. A single dose administration of the phage cocktail to birds of age 24 days, 4 days post *Campylobacter* infection significantly reduced *C. jejuni* caecal counts, throughout the experiment, compared to mock-treated birds. The largest reduction, of 2.4 $\log_{10}$ CFU g^{-1} in the caeca was observed 2 days post treatment, compared with control birds. After the 2 days, *C. jejuni* counts increased in the phage-treated birds but numbers remained significantly lower than those observed in the control birds, by 1.3 $\log_{10}$ CFU g^{-1} after 5 days. Phage treatment proved effective in the ileum and the colon but was most effective in the caecum as this organ harboured the highest *C. jejuni* counts. The phages were proved to co-exist in the birds, avoiding competitive

exclusion, as they both replicated *in vivo*, and the titre recovered remained stable for both phages. Resistance was observed, after treatment, in only ~10% of *C. jejuni* HPC5 isolates in the phage-treated group. Furthermore, predation of *C. jejuni* by the phages was not recorded to affect the microbiota of the chickens and only selectively reduced *C. jejuni* numbers. The results outlined in this study offer further confirmation of the efficacy of phage treatment in reducing *Campylobacter* colonisation in broilers.

An investigation on broiler farms in the northwest of Germany by Kittler *et al*., (2013), examined the effect of a bacteriophage cocktail application on *C. jejuni* counts in commercial broiler flocks. This bacteriophage application was previously effective in non-commercial studies, under controlled experimental conditions, so the aim was to test it under commercial conditions. A phage cocktail was applied to broilers on three commercial farms, which consisted of a control and an experimental group, upon confirmation of successful *Campylobacter* colonisation. The cocktail was applied to birds via drinking water in experimental groups. Findings suggested that phage application against *Campylobacter* in commercial broilers can lead to reductions of up to 3.2 $\log_{10}$ CFU, which provides a promising future for public health. To obtain maximum reductions, application was suggested to occur 2 to 4 days before slaughter and broad-spectrum phage cocktails are essential to target different host strains.

Overall, these studies outline the proven therapeutic success of bacteriophages in reducing *Campylobacter* counts in broiler chickens, to combat the ongoing, high rates of *Campylobacter* infection in humans worldwide. The selection of appropriate phages (in terms of their host ranges and other factors), their optimum dosage and the optimum time frame of phage application prior to slaughter, are pivotal in the success of this therapy (Loc-Carrillo *et al.,* 2005), to experience reductions in the carriage of campylobacters in poultry and corresponding reductions in community caseloads, improving consumer health.

1.9. Aims of the study

The biocontrol of food-borne pathogens present in domestic farm animals has been studied extensively, using various methods. Control through bacteriophage therapy has shown major success in recent years, using various monophages and bacteriophage cocktails. This study aimed to assess the efficacy of one bacteriophage cocktail: CampyShield (Intralytix, USA), against *Campylobacter* spp. in the caeca and liver of broiler chickens. Efficacy was determined using various characteristics such as testing host ranges and infection of resistant strains with respect to their EOP. The impact of this study assists in discovering a successful method to reduce contamination in poultry, and therefore, food-borne diseases, to improve consumer health.

Chapter 2- Materials and methods

2.1. Solutions

2.1.1. Magnesium sulphate ($MgSO_4$)

A 1 M solution of $MgSO_4$ (Sigma-Aldrich) was prepared by dissolving 12 g $MgSO_4$ in 100 ml reverse osmosis (RO) water, sterilised by autoclaving and stored at room temperature (20-22 °C). When suspending *Campylobacter* cells, a 10 mM $MgSO_4$ solution was prepared by adding 100 µl 1 M stock to 10 ml sterile RO water.

2.1.2. Maximum Recovery Diluent (MRD)

The MRD medium (CM0733, Oxoid, Basingstoke, UK) was dissolved in RO water (9.5 g/l) and sterilised by autoclaving according to the manufacturer's instructions. It contained the following ingredients: Peptone 1.0 g/l, Sodium chloride 8.5 g/l, pH 7.0 and was used in serial dilutions and for liver homogenates and bacterial suspensions. When suspending *Campylobacter* cells, 10 ml MRD was used for half a plate of culture.

2.1.3. Salt Magnesium (SM) Buffer

The SM buffer was obtained from bio-WORLD (41920012-1, USA) and stored at room temperature. It contained 50 mM Tris-HCl, pH 7.5, 100 mM Sodium chloride, 8 mM Magnesium sulphate, 0.01 % gelatin, pH 7.4-7.6. It was used for serial dilutions and bacterial suspensions.

2.2. Culture media

2.2.1. Bolton Broth

Bolton broth (CM0983, Oxoid, Basingstoke, UK) contained the following ingredients g/l: Meat peptone 10.0; Lactalbumin hydrolysate 5.0; Yeast extract 5.0; Sodium chloride 5.0; Alpha-ketoglutaric acid 1.0; Sodium pyruvate 0.5; Sodium metabisulphite 0.5; Sodium carbonate 0.6; Haemin 0.01; pH 7.4. The broth base (13.8 g) was dissolved in 500 ml of RO water and sterilised by autoclaving according to manufacturer's instructions. Bolton broth selective supplement (1.00079, Millipore, Germany), contained (per vial) Vancomycin 10 mg; Cefoperazone 10 mg;

Trimethoprim 10 mg; Amphotericin B 5 mg. This was prepared by dissolving the lyophilisate in the original vial by adding 5 ml of a 50:50 mixture of sterile distilled water and ethanol, using a swirling motion. The contents of a vial (5 ml) was aseptically added to 500 ml of sterile Bolton Selective Enrichment Broth (Base) and mixed.

2.2.2. Mueller-Hinton (MH) Broth

The MH broth (CM0405, Oxoid, Basingstoke, UK) contained the following ingredients g/l: Dehydrated infusion from beef 300.0; Casein hydrolysate 17.5; Starch 1.5; pH 7.3. The broth base (10.5 g) was dissolved in 500 ml of RO water and sterilised by autoclaving according to manufacturer's instructions.

2.2.3. MH agar

Agar Bacteriological (Agar No.1; LP0011B, Oxoid, Basingstoke, UK) was added to MH broth (**2.2.2.**) to give 1.2 % w/v for sub-culturing, or 0.4 % w/v for motility testing and sterilised by autoclaving according to the manufacturer's instructions. The medium was then mixed thoroughly and poured into Petri dishes.

2.2.4. Blood agar (BA)

Blood agar base No.2 (Oxoid, Basingstoke, UK) contained the following ingredients g/l: Proteose peptone 15.0; Liver digest 2.5; Yeast extract 5.0; Sodium chloride 5.0; Agar 12.0; pH 7.4. 20 g was added to 500 ml of RO water, then sterilised by autoclaving according to manufacturer's instructions. The medium was kept at 50 °C until needed, then it was cooled to 45 °C. Defibrinated horse blood (Scientific Laboratory Supplies, UK) was added (25 ml to 500 ml medium). The medium was then mixed thoroughly and poured into Petri dishes. For sub-culturing multiple colonies on a single plate, (to prevent *Campylobacter* from swarming), extra Agar No.1 was added to the base powder to give 2 % w/v.

2.2.5. New Zealand casamino acid yeast extract sodium chloride and magnesium sulphate (NZCYM) basal agar

The NZCYM broth (N3643, Sigma-Aldrich, Gillingham, UK) contained the following ingredients g/l: Casamino acids 1.0; MgSO4 0.98; Sodium chloride 5.0; Tryptone 10.0; Yeast extract 5.0; pH 6.8-7.2. The broth base (11.0 g) and 6.0 g Agar No.1 (LP0011B, Oxoid, Basingstoke, UK) to give 1.2 % w/v, were prepared in 500 ml of RO water and sterilised by autoclaving according to the manufacturer's instructions.

2.2.6. NZCYM overlay agar

The NZCYM agar recipe (**2.2.5.**) was modified by reducing the final concentration of NZCYM base to 0.6 % (w/v) and sterilised by autoclaving according to the manufacturer's instructions. It was then stored at 50 °C until needed, when it was cooled and dispensed into 5 ml amounts for the addition of bacterial suspensions, to create lawns. Overlay agar suspensions were then poured over NZCYM bottom plates for obtaining phage plaques.

2.2.7. Modified Charcoal Cafoperozone Deoxycholate (mCCDA) agar

Campylobacter blood-free selective agar base (CM0739, Oxoid, Basingstoke, UK) contained the following ingredients g/l: Nutrient broth no. 2 25.0; Bacteriological charcoal 4.0; Casein hydrolysate 3.0; Sodium deoxycholate 1.0; Ferrous sulphate 0.25; Sodium pyruvate 0.25; Agar 12.0; pH 7.4. The base (22.75 g) was prepared in 500 ml of RO water and sterilised by autoclaving according to manufacturer's instructions. It was stored at 50 °C until needed, when it was cooled and 3 ml of prepared CCDA selective supplement antibiotic (1 vial) (SR0115, Oxoid, Basingstoke, UK) was added and mixed into the sterile agar base, according to the manufacturer's instructions, before pouring into Petri dishes. The CCDA selective supplement antibiotic contained (per vial): Cefoperazone 16 mg; Amphotericin B 5 mg. For enumeration, (to prevent *Campylobacter* from swarming), extra Agar no.1 was added to the base powder to give 2 % w/v of agar.

2.3. Bacterial strains and their storage

Campylobacter reference strains are shown in Table 3. Subcultures of these strains were prepared from stocks stored at -80 °C. The contents were thawed and used to inoculate fresh BA (**2.2.4.**) plates, which were then incubated under microaerobic

conditions (**2.5.1.**) at 42 °C for 18-24 h. When required, strains were subculutred on BA and incubated for 18-24 h at 42 °C under microaerobic conditions. They were stored in gas jars under microaerobic conditions at 4 °C.

Table 3. Bacterial strains used. Names, reference number and source of bacterial strains used in experiments. NCTC = National Collection of Type Cultures (Colindale, London, UK).

***Campylobacter* species**	**Reference number**	**Source**	**Sequence (Accession)**
C. jejuni	NCTC 12662	NCTC	NZ_CP019965
C. jejuni	GIIC8	Conventional barn-reared UK broiler chicken flocks	N/A
C. jejuni	PT14	NCTC	NC_018709
C. coli	NCTC 12668	NCTC	N/A

Colonies observed on BA and CCDA (**2.2.7.**) conformed to the description of typical *Campylobacter* growth on these media. Typical colonies were defined as grey/white, circular, flat and 1-2 mm in diameter. They were also convex, entire, glistening and moist. No further tests were performed.

2.4. Bacteriophages and their storage

Bacteriophage stocks were stored in SM buffer (**2.1.3.**) at 4 °C. CampyShield (Intralytix, USA) is a phage cocktail that was used in the Broiler chicken trial (**2.6.**); the monophages that make up the cocktail are shown in Table 4.

Table 4. Phages used in the trial and this study. The main *Campylobacter* host for the propagation of each phage is shown, along with the source, group and DNA sequence/Accession.

	Bacteriophage	Source	Propagating strain	Group	Sequence (Accession)
	CP30A	Conventional barn-reared UK broiler chicken flocks	*C. jejuni*	III	NC_018861
CampyShield	CJLB-5	Intralytix, USA	*C. jejuni*	III	MW057932
	CJLB-10			III	MW074124
	CJLB-14			II	MW074126

2.5. Growth and storage of campylobacters

2.5.1. Microaerobic atmosphere generation

After subculturing, colonies were routinely grown under microaerobic conditions of 5 % O_2, 2 % H_2, 5 % CO_2, 85 % N_2, 42 °C, either in the Whitley M35 Variable Atmosphere Workstation (Don Whitley Scientific, UK) or in a 2.5 L AnaeroGen gas jar (Oxoid, UK), using the evacuation/replacement method. This method involved evacuating the jars to -20 Hg, using a vacuum, and replacing it to 0 Hg, with an anaerobic gas mixture (85 % N_2, 10 % CO_2 and 5 % H_2). It is important to note that these two methods may provide subcultures with different gaseous environments to each other, as the M35 Workstation is a more controlled environment. This could lead to variation in results.

2.6. Broiler chicken trial

2.6.1. Summary

The 'Broiler *Campylobacter* challenge test' was conducted under UK HO licence at Drayton Animal Health (https://www.draytonah.co.uk/) on Ross 308 broiler chickens, which involved infecting them with *Campylobacter* to investigate the

effect of different phages on *Campylobacter* caecal colonisation. There were five trial groups of chickens, consisting of eight pens each, with four birds allocated at random in each pen. Each group was given a different treatment, shown in Table 5; challenged birds were infected with *C. jejuni*. *Campylobacter* colonisation was enumerated in all treatment groups, on CCDA, as were phage counts on lawns. Post-trial phage-resistant isolates were then obtained and subjected to further testing in the trial and in this study. Livers for this study were obtained aseptically from trial birds in each pen of the five groups and frozen at -80 °C until needed.

2.6.2. Study design

Table 5. Treatment details given to the chickens in each trial group T1-T5.

Treatment group	**Pen numbers**	**Treatment description**	**Dose of phage ($PFUml^{-1}$)**	**Days receiving phage**	***Campylobacter* challenge day 21 and day 32 ($CFUml^{-1}$)**	**Target number of birds Day 0**
T1	1-8	No challenge No phage	-	-	-	32
T2	9-16	No challenge CampyShield	10^9	39 & 40	-	32
T3	17-24	Challenge No phage	-	-	10^7	32
T4	25-32	Challenge CP30A phage	10^9	39 & 40	10^7	32
T5	33-40	Challenge CampyShield	10^9	39 & 40	10^7	32

2.6.3. Isolating phage-resistant caecal isolates from group T5

Upon *Campylobacter* enumeration on CCDA, ~100 colonies per pen (16 per bird) in groups T3, T4 and T5 were subcultured onto 2 % BA and incubated. These plates were stored under microaerobic conditions at 4 °C until needed. Out of 16, 12 colonies were selected for sensitivity testing to experimental phages. These isolates

were individually subcultured onto BA and used to prepare bacterial lawns for CampyShield and CP30A phage testing. Treatment phages were subjected to a dilution series up to 10^{-7} and 10 µl aliquots of dilutions 10^{-2}- 10^{-7} were dispensed, in triplicate, onto lawns of each post-trial isolate selected. Plates were left to dry and inverted for microaerobic incubation for 18-24 h. Lawns of fully sensitive isolates were expected to observe approximately 1 PFU per 10 µl at 10^{-7}. After incubation, plaques were counted and the titre was determined. The titre was divided by the control titre to calculate the efficiency of plating (EOP) values. An EOP value of >0.01 was determined as sensitive and <0.01, as insensitive to phage. Insensitive isolates were then prepared in storage solution (MH broth with 15 % sterile glycerol added) and kept at -80 °C until needed for further testing.

2.7. Isolating *Campylobacter* from trial chicken livers

Several attempts were made at isolating *Campylobacter* spp. from broiler chicken livers in trial groups T1-5. Livers were obtained from -80 °C and thawed on ice. Pieces were cut off using a sterile, disposable scalpel and weighed in a stomacher bag. MRD was added to each bag to make 10 % w/v and stomached for 60 s on medium, for homogenisation, using the Seward Stomacher® 80 lab system. Homogenates were inoculated, using a swab, onto mCCDA agar. Plates were then incubated at 42 °C under microaerobic conditions for 18-24 h and examined for typical colonies.

2.7.1. Enrichment technique I (CCDA selective supplement)

This first method was used for livers in groups T3 and T4. Bijous were made up of 4 ml stomached homogenate and enrichment broth containing: 4 ml Bolton broth (**2.2.1.**) 200 µl lysed, defibrinated horse blood and CCDA selective supplement (final concentration Cefoperazone 32 mg/l and Amphotericin B 10 mg/l). Bijous were incubated at 37 °C for 48 h. Samples were then cultured onto mCCDA agar and incubated at 42 °C under microaerobic conditions for 18-24 h.

2.7.2. Enrichment technique II (Bolton broth selective supplement)

This second method was used for group T2 and T5 livers and used a more enriched broth. Bijous were made up of 4 ml stomached homogenate and 4 ml enrichment broth of 2x strength, containing Bolton broth, laked horse blood (5 % final concentration) and Bolton broth selective supplement (**2.2.1.**; at 2x strength). Bijous were incubated at 37 °C for 48 h.

2.7.3. Increased sensitivity (*Campylobacter*) technique

The last method was used on group T2, T4 and T5 livers. Livers were obtained from -80 °C and thawed. Chunks were cut off using a scalpel and weighed in a stomacher bag. Enrichment broth contained: 500 ml Bolton broth (**2.2.1.**; single strength), prepared with the addition of laked horse blood (5 % v/v) and Bolton broth selective supplement, which was added to each bag to make 10 % w/v. Bags were then stomached for 60 s on medium and heat sealed (to prevent leakage and reduce headspace) for incubation at 37 °C for 48 h. Homogenates were collected in universals and cultured onto mCCDA agar to be incubated under microaerobic conditions at 42 °C for 18-24 h.

2.8. Isolating phage from trial chicken livers

Several attempts were made at isolating bacteriophage from broiler chicken livers in all trial groups T1-5. Refer to section **2.7.** for the method used to obtain liver homogenates (in single strength Bolton broth with laked blood and Bolton selective supplement). To increase the possibility of isolating phages, *Campylobacter* host suspensions were added to liver homogenates. The suspensions were made by swabbing half a plate of bacteria and adding this to 10 ml MRD (**2.1.2.**). Aliquots of this suspension (0.25 ml) were added to each liver homogenate sample and incubated for 24 h at 42 °C. These are the optimum conditions for actively growing *Campylobacter* as compared to the lower, longer incubation applied to recover damaged cells, following freezing. Aliquots (1 ml) of the liver homogenate with added *Campylobacter* were filtered through 0.2 µm filters to remove bacteria but allow phage to pass. Aliquots of 10 µl of eluate were dispensed in triplicate onto bacterial lawns (**2.8.1.**). Droplets were then left to dry into the agar and inverted for microaerobic incubation at 42 °C for 18-24 h. Plates were examined for plaque formation.

2.8.1. Preparing the bacterial host lawns

Bacterial host strains were grown on BA (**2.2.4., 2.3.)** and were harvested by swabbing and suspending half a plate of subculture into 10 ml of 10 mM $MgSO_4$ (**2.1.1.**). Overlay agars (**2.2.6.**) were then made by adding 500 µl of bacterial suspensions to 5 ml molten NZCYM overlay agar, which had been cooled from 50 °C for 5-10 min. The inoculated overlay agar was then immediately poured onto NZCYM basal agar plates (**2.2.5.**) and rotated gently to produce an even lawn for setting at room temperature.

2.8.2. Phage isolation

Samples of unincubated liver homogenate, incubated enrichment broth with and without the added host (**2.7.**) were filtered and dispensed as 10 µl droplets onto the freshly prepared host lawns and incubated at 42 °C for 18-24 h.

2.8.3. Propagating Group T4 and T5 liver phages on *C. jejuni* strains.

Isolated plaques were picked off overlay agar plates, using the cut off tip of a sterile 1000 µl- capacity micropipette and suspended in 100 µl SM buffer. Eluates were then dispensed as 10 µl aliquots onto lawns of *C. jejuni* NCTC 12662 and *C. jejuni* PT14. Plates were then inverted and incubated under microaerobic conditions at 42 °C for 18-24 h.

2.9. Bacteriophage titrations

Detection limits and titrations were carried out on Group T3 and T4 liver samples and on CP30A, CJLB-5, CJLB-10, CJLB-14 and CampyShield phages (original stocks were $10^{8/9}$ PFU ml^{-1}). A ten-fold serial dilution series in triplicate in SM buffer (**2.1.3.**) was prepared for each titration. Following this, 10 µl of samples at 10^{-2} to 10^{-6} were dispensed, in triplicate, onto bacterial lawns of *C. jejuni* PT14 and/or *C. jejuni* NCTC 12662 and left to dry. Plates were incubated under microaerobic conditions at 42 °C for 18-24 h. The number of plaque forming units (PFU) per ml of the bacteriophage suspension was determined.

2.10. 'Spike' experiment to establish limit of detection of phages

Three group T3 livers (*Campylobacter* control birds not treated with phage during the trial and that tested negative for phages) from birds P22, P23 and P24 (birds were numbered based on their pen allocation, as seen in Table 5) were obtained from -80 °C and thawed on ice. Refer to section **2.7.** for homogenisation method. The whole homogenate contents from these livers were added together and mixed thoroughly to provide sufficient 'mock' samples to do the experiment. A 5 ml aliquot of the mixed homogenates was added to each of 15 bijous. A ten-fold dilution series of CP30A (approx. 10^8 PFU ml^{-1}) to 10^{-6} was prepared. Aliquots of 10 µl of dilutions 10^{-3}-10^{-6} to give approximately 200, 20, 2 and 0.2 phages/bijoux were added to three bijous each, containing homogenates. Three bijous were left as controls. A 1 ml aliquot of each of 15 homogenates was then added to Eppendorfs and stored at 4 °C until needed, these were labelled 'Direct'.

Half a plate of *C. jejuni* PT14 was added to 10 ml MRD and Bolton Enrichment broth at 2x strength (**2.2.1.**). Four ml of the enrichment broth/*Campylobacter* suspension was then added to all bijous, which were incubated at 42 °C for 24 h. A 1 ml aliquot of each bijoux was then filtered through a 2 µm filter to remove bacteria and labelled 'Enrichment'. 'Direct' samples (1 ml) were also filtered through a 2 µm filter. Lawns of *C. jejuni* PT14 were made and all 30 samples were dispensed in triplicate as 10 µl droplets onto lawns and left to dry then inverted and incubated at 42 °C under microaerobic conditions for 18-24 h. Plaques were then observed to calculate the limit of detection. Figure 5 below displays a flow diagram summarising this procedure.

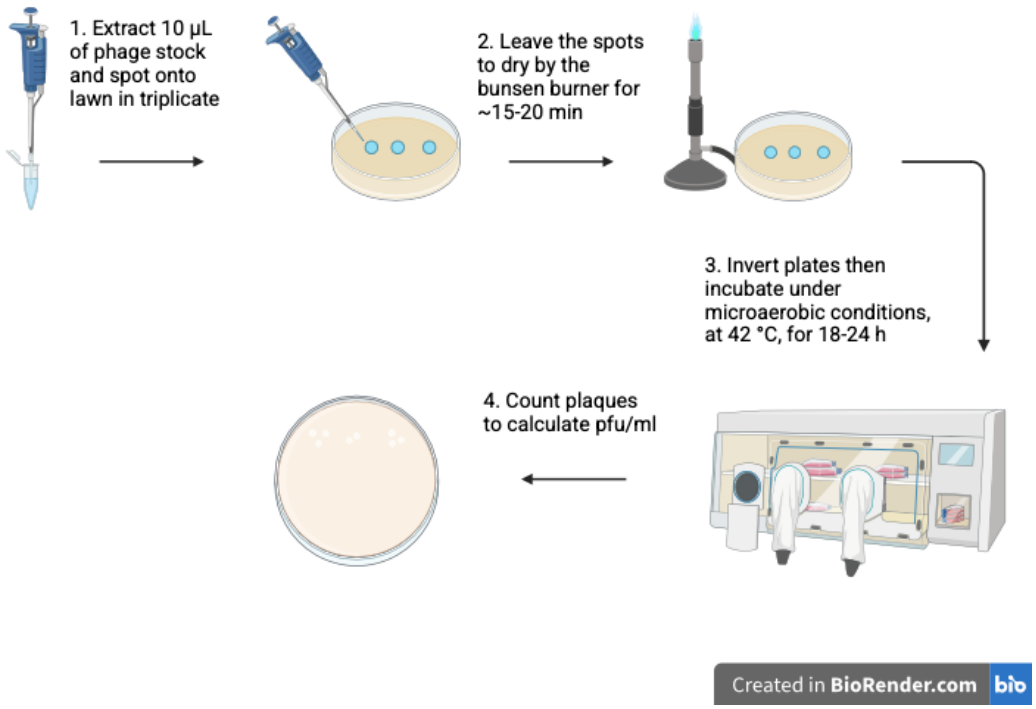

Figure 5. Flow diagram showing how bacterial lawns were used to identify phage presence.

2.11. CampyShield and liver phage testing on *Campylobacter* strains

Phages isolated from livers P35, P38 and P40, along with CJLB-5, CJLB-10, CJLB-14 monophages and the CampyShield cocktail were all subjected to testing on different *Campylobacter* strains. Strains included: *C. jejuni* PT14, *C. coli* NCTC 12668 and *C. jejuni* GIIC8. Lawns of all strains were made and CJLB-5, CJLB-10, CJLB-14 and CampyShield phages were each subjected to a 1 in 10 serial dilution series with SM buffer, the initial stock was $10^{8/9}$ PFU ml^{-1}. Droplets (10 µl) of dilution samples at 10^{-2} to 10^{-6} were dispensed in triplicate onto lawns of each strain. The P35, P38 and P40 phages were dispensed directly onto lawns of each strain, in triplicate and left to dry. Plates were incubated under microaerobic conditions at 42 °C for 18-24 h.

2.12. Testing for the presence of CJLB-14 phage in chicken livers

Group T2 and T5 liver homogenates (**2.7., 2.8.2.)** were prepared. A 0.25 ml suspension of *C. coli* NCTC 12668 (approximately 10^9 CFU ml^{-1}) in MRD was added to each bag prior to stomaching. Bags were then heat sealed and incubated at 42 °C for 18-24 h for filtering and dispensing onto lawns of *C. coli* NCTC 12668. CJLB-10 was also dispensed as a control. Plates were then inverted and incubated under microaerobic conditions at 42 °C for 18-24 h, for plaque observation.

2.13. Isolating phage from group T2 caecal contents

Eight group T2 caecal isolates (phage control birds with no *Campylobacter* colonisation) were obtained from -80 °C storage and partially thawed on ice for 30-45 min. Approximately 0.1 g of contents was extracted and placed into a bijoux. Bolton Enrichment broth (7 ml; **2.2.1.**) was then added to each bijoux. Host bacteria *C. jejuni* PT14, at approximately 10^8 CFU m^{-1} in MRD was added to each bijoux containing enrichment broth and caecal contents. Bijous were then incubated at 42 °C for 18-24 h for filtering and dispensing onto *C. jejuni* PT14 lawns. Plates were then inverted and incubated under microaerobic conditions at 42 °C for 18-24 h. Following incubation, results on plaque formation were recorded.

2.14. Group T5 resistant caecal isolates

2.14.1. Testing resistance to CampyShield phages and CP30A

A group of 16 caecal isolates, from group T5 birds, identified as being resistant to CampyShield phages were subcultured on BA. Lawns were then made using each isolate. CampyShield, CJLB-5, CJLB-10 and CP30A, all at 10^6 PFU ml^{-1}, and CJLB-14 at 10^9 PFU ml^{-1} were then dispensed in 10 µl aliquots, in triplicate, directly onto these lawns and left to dry, then inverted for microaerobic incubation at 42 °C for 18-24 h. Resistance and sensitivity to the different phages was recorded and further investigations were conducted.

2.14.2. Motility testing

Colonies from BA plates of each resistant caecal isolate were extracted using a loop and inoculated onto the centre of 0.4 % w/v MH agar plates. Plates were then incubated under microaerobic conditions for 48 h. Motility was measured using a ruler, by recording the distance from the centre of the inoculated spot to the edge of the ring formed by the colony. Isolates were compared with a *C. jejuni* PT14 control and recorded as motile, partially motile or non-motile.

2.14.3. Identifying carrier state phage

A group of 14 caecal isolates identified as being resistant to CampyShield phages were subjected to carrier state testing. Colonies from each strain were swabbed and emulsified in 1 ml SM buffer. Each suspension was then filtered through a 0.2 µm filter into a new Eppendorf. One 10 µl spot from each isolate suspension was dispensed onto a *C. jejuni* PT14 lawn and a *C. coli* NCTC 12668 lawn. Plates were then incubated under microaerobic conditions at 42 °C for 18-24 h.

2.14.4. DNA extraction

DNA was extracted from 7 isolates identified as being resistant to CampyShield phages and were chosen based on their interaction with different phages and their motility results. Cells from BA subcultures were suspended in 1 ml sterile RO water. All bacterial genomic DNA was then isolated using the GenEluteTM Bacterial Genomic DNA kit (Sigma-Aldrich, USA), alongside the protocol as described (Sigma-Aldrich, 2024). In short, the bacterial RO suspensions were centrifuged at 12,000 x g for 2 min at room temperature using a Sigma 1-16k benchtop centrifuge (Sigma-Aldrich). Next, the pellet was suspended in 180 µl of Lysis Solution T (BB6678) and 20 µl of RNase A solution (R6148) was added for subsequent incubation at room temperature for 2 min. A 20 µl aliquot of 20 mg/ml Proteinase K (Sigma-Aldrich) stock solution was then added and the mixtures were incubated for 30 min at 55 °C. Next, 200 µl of Lysis Solution C (B8803) was added to each sample and the mixtures were vortexed for 15 seconds, until homogenised mixtures were obtained, mixtures were then incubated at 55 °C for 10 min. Columns were prepared by adding 500 µl of Column Preparation Solution (C2112) for centrifugation in a Sigma 1-16k benchtop centrifuge at 12,000 x g for 1 min. The eluates were discarded. An aliquot of 200 µl of 100 % ethanol (Sigma-Aldrich) was added to each

lysate sample and mixed thoroughly by vortexing for 5-10 seconds. This was then added onto the prepared column, which was centrifuged at 6,500 x g for 1 min. The eluate and collection tubes were discarded and all columns were put into new collection tubes. Next, 500 µl of Wash Solution 1 (W0263) was added to the columns and all the tubes were centrifuged again for 1 min at 6,500 x g. The collection tubes were then discarded again and replaced and 500 µl of Wash Solution 1 was added to the column for a second wash. The columns were then centrifuged at maximum speed for 3 min to dry the column. The collection tube was discarded and replaced. The DNA was eluted by adding 200 µl of Elution Solution (B6803: 10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) directly to the centre of the each column. Tubes were then centrifuged at 6,500 x g for 1 min for collection of the eluted DNA.

2.14.5. Measuring the purity of DNAs

The Nanodrop ND-1000 spectrophotometer (supplied by Labtech) was used to measure the purity and concentration of DNAs. Isolates were then stored at 4 °C until they were needed for sequencing.

2.14.6. DNA Sequence Library Preparation

The DNA sequence determination was carried out by Deep Seq (https://www.nottingham.ac.uk/deepseq/). A total of 200 ng of DNA was used for sequencing library preparation. Indexed sequencing libraries prepared using the Nextera DNA Prep Library Prep Kit (Illumina) and IDT® for Illumina® DNA/RNA UD Indexes Set D (Illumina;). For all samples, 5 cycles of PCR were used. The library was quantified using the Qubit Fluorometer and the Qubit dsDNA HS Kit (ThermoFisher Scientific). Library fragment-length distribution was assessed using the Agilent TapeStation 4200 and the Agilent D1000 ScreenTape Assay (Agilent). Final library quantification was performed using the KAPA Library Quantification Kit for Illumina (Roche). The library pool was then sequenced on an Illumina MiSeq using the MiSeq Reagent Kit v2 (500 cycle; Illumina) to generate 250-bp paired-end reads. The reads were mapped to the genome sequence of wild type *C. jejuni* PT14 (CP003871; Brathwaite *et al*., 2013) or wild type NCTC 12662 (CP019965; Gencay *et al*., 2017) and any nucleotide variants detected at > 50% were recorded (Genomics Workbench 20.0.3).

2.15. Attempts to isolate resistant *C. jejuni* NCTC 12662 *in vitro*

2.15.1. Growth curve experiment

Growth experiments using *C. jejuni* NCTC 12662, incubated with CampyShield phages and with the monophages in the cocktail were carried out to generate resistant *Campylobacter* strains. Half a plate of *C. jejuni* NCTC 12662 was suspended in 10 ml MRD. Three 'Infected' and three 'Control' cultures were made for each phage stock and added to conical flasks or universals. Infected cultures contained: 15 ml MH broth, 10 µl *C. jejuni* NCTC 12662 suspension of approximately 10^{7-8} CFU ml^{-1} and 100 µl CJLB-5 stock only or 100 µl CJLB-10 stock only or 100 µl CJLB-14 stock only, at approximately 10^{7} pfu ml^{-1}. Control cultures contained: 15 ml MH broth and 10 µl *C. jejuni* NCTC 12662 suspension of approximately 10^{7-8} CFU ml^{-1}. Cultures were placed in an AnaeroGen gas jar and subjected to microaerobic conditions then placed into a shaking incubator at 42 °C, 120 rpm. Samples (1 ml) were taken from all cultures at the following time points and dispensed into Eppendorfs: T = 0 h, T = 2 h, T = 4 h, T = 6 h and T = 24 h. After samples were obtained, cultures were placed back in the AnaeroGen gas jar, under microaerobic conditions, and returned to the shaking incubator. At each time point, Infected samples were enumerated for *Campylobacter* and phage and Control samples for *Campylobacter* only.

The Miles and Misra technique (as described by Datta, 2021) was used to enumerate *Campylobacter*; samples were subjected to a 1 in 10 serial dilution series with MRD. Five x 10 µl aliquots of each dilution from 10^{-2} to 10^{-7} were dispensed onto 2 % mCCDA agar (to obtain single colonies) and incubated under microaerobic conditions at 42 °C for 48 h. Colonies isolated from infected T = 24 h plates were then subcultured onto BA, incubated, and used to make lawns for CampyShield monophages, to test for bacterial resistance.

The *C. jejuni* NCTC 12662 host was used to make lawns to enumerate phage in infected samples. Samples were subjected to a 1 in 10 serial dilution series with MRD. Aliquots of 5 x 10 µl from each dilution of 10^{-1} to 10^{-3} were dispensed onto lawns and incubated under microaerobic conditions at 42 °C for 18-24 h.

After dispensing the CampyShield monophages on the T = 24 h lawns (**2.14.1.**), DNA was extracted (**2.14.4.**) and the DNA sequences were determined (**2.14.6.**) from 4 bacterial isolates that exhibited resistance to these phages.

Chapter 3- Investigating campylobacters recovered post phage therapy

3.1. Introduction

Campylobacter spp., *C. jejuni* in particular, is generally considered to colonise the avian gut as a commensal organism due to infection of birds *via* the faecal-oral route; broilers are often regarded as natural hosts for this pathogen. Upon ingestion, *Campylobacter* colonises the intestinal tract and ingestion of numbers as low as 35 CFU allows them to reach the caecum and replicate, resulting in successful colonisation of chickens within 24 hours. Colonised broiler chickens harbour high *Campylobacter* counts in their caeca; up to 10^9 CFU per gram of caecal contents. Once colonisation is detected among one broiler, > 95 % of the birds in that flock are colonised within a few days and remain colonised until slaughter (Hermans *et al*., 2011). Over time, *C. jejuni* and other *Campylobacter* spp. have evolved several survival and colonisation mechanisms, allowing them to continually adapt to their chicken host and persistently colonise chicken caeca at high levels. Hermans *et al*., (2011), states that this persistence of *C. jejuni* in the chicken GI tract indicates that it may possess regulatory systems that enable protection towards hostile environments inside and outside the host. Although there have been technological advances reported to reduce the colonisation of chicken caeca, such as through the use of bacteriophage therapy and vaccines, it is still vital to develop these methods for commercial applications. This chapter investigates the properties of bacteriophages and campylobacters recovered from the caeca and the post-phage therapy application of *Campylobacter* colonised broiler chicken caeca using CampyShield.

The 'Broiler *Campylobacter* challenge test' (**2.7.**) investigated the effect of different phages on the caecal colonisation of broilers. Broiler chickens were infected with *Campylobacter jejuni* and treated with CampyShield phages (Table 4) or CP30A. The phage CP30A was selected as a positive control as it has a proven track record in phage therapy against *Campylobacter*, with a broad host range for target bacteria, it lacks host virulence-associated genes and it has been demonstrated to be successful in reducing *C. jejuni* counts both *in vivo* and *in vitro*, without affecting non-target bacteria (Richards *et al*., 2019). When assessing the therapeutic success of a new

phage/phage cocktail, its stability is one of the crucial variables to consider, and CP30 has proven to be robust and suitable for international transport as lyophilized and spray dried formulations (Liang *et al*., 2020). Therefore, comparing CampyShield with CP30A offers an insight into the extent of success CampyShield may offer against campylobacters in chickens.

Post-trial *Campylobacter* isolates were then subjected to phage-sensitivity testing. Single colony isolates were obtained from groups T3 and T5 and were tested for their sensitivity to the CampyShield cocktail. All T3 isolates remained sensitive to phages but there was a high number of phage-insensitive T5 isolates. Single colony isolates were also obtained from group T4 post treatment and tested for their sensitivity to CP30A. Several colonies were insensitive to CP30A, but a much smaller proportion compared to those resistant to CampyShield in group T5. These resistant isolates were stored at -80 °C until subjected to further resistance testing in attempt to determine the reason for resistance.

3.2. Results

3.2.1. Summary of post-trial sensitivity testing of caecal isolates to CampyShield phages and CP30A

Campylobacter enumeration of caecal contents (CFU per gram) was carried out for all treatment groups; T3-T5 produced colonies on CCDA. ~100 colonies from groups T3-T5 (16 colonies per bird) were individually subcultured on BA and 12/16 were obtained for lawn preparation and phage testing. EOP values were then obtained and the isolates 1-12 were designated sensitive or insensitive to CampyShield or CP30A.

All T3 isolates were sensitive to CampyShield, as they had not been exposed to the phages. However, 65 % of T5 isolates were insensitive to CampyShield; 12 colonies from each bird P33-40 were tested. Generally, higher *Campylobacter* counts per gram of caecal contents corresponded to low phage sensitivity, based on the 12 colonies tested; the bird with the highest *Campylobacter* count of 8.2 $\log_{10}$ CFU g^{-1} gave an 8 % sensitivity value and the bird with the lowest count of 4.8 $\log_{10}$ CFU g^{-1} gave a phage sensitivity of 75 %. Therefore, the higher the *Campylobacter* count, the more resistant isolates were present. Phage insensitivity of the isolates ranged from 25 % to 92 %, with 5 birds harbouring a phage insensitivity value of above 65 %. By

contrast, upon testing of T4 isolates to CP30A, only 24 % (23/96) exhibited insensitivity to this phage. Two of the 12 resistance isolates from each T5 bird were obtained from -80 °C, thawed, and subcultured for further testing.

3.2.2. Phage enumeration of group T2 caecal contents

Three attempts were made at isolating phage from the caecal contents of group T2 birds. Eight group T2 caecal isolates, P9-16, were obtained from -80 °C storage and subjected to enrichment with *C. jejuni* PT14, before application onto *C. jejuni* PT14 lawns. Results obtained were negative; no phage was isolated from any of these samples.

3.2.3. Further post-trial sensitivity testing of group T5 resistance isolates

Approximately 10^5 PFU of CampyShield or the monophages CJLB-5, CJLB-10, CJLB-14 and CP30A were applied as 10 µl droplets directly onto lawns of sixteen resistant isolates to ascertain the range of the phage resistance. Plaques were observed and resistance/sensitivity of the isolates was recorded. The results are shown in Table 6. All isolates were resistant to the CampyShield cocktail and all but P39/2 were resistant to CJLB-5 and CJLB-10. Six isolates were resistant to CJLB-14 (38 %), but no isolates were resistant to CP30A. These results indicate that CampyShield may not represent an effective cocktail to reduce caecal colonisation by *C. jejuni* of broiler chickens.

Table 6. Sensitivity/resistance testing of group T5 resistant caecal isolates against CampyShield phages and CP30A.

	Phage type				
Caecal isolate	**CampyShield cocktail**	**CJLB-5 (gIII)**	**CJLB-10 (gIII)**	**CJLB-14 (gII)**	**CP30A**
P33/1	R	R	R	R	~L
P33/2	R	R	R	R	L
P34/5	R	R	R	R	L + P
P34/6	R	R	R	R	~L
P35/1	R	R	R	~L	~L + P
P35/2	R	R	R	~L	~L + P
P36/3	R	R	R	~L	~L + P
P36/4	R	R	R	L	P
P37/1	R	R	R	L	~L + P
P37/2	R	R	R	~L	P
P38/1	R	R	R	L	~L + P
P38/2	R	R	R	L	~L + P
P39/2	R	P	P	R	~L + P
P39/9	R	R	R	R	~L + P
P40/1	R	R	R	L	P
P40/2	R	R	R	L	L + P

P, plaque formation; L, lysis; ~L, limited lysis (showing overgrowth); R, resistance.

3.2.4. Motility testing

The sixteen resistant isolates were then subjected to motility testing by inoculating 0.4 % MH agar with each isolate. The distance from the centre of the inoculum to the edge of the ring formed (radius) was measured and isolates were categorised motile, partially motile or non-motile. Measuring motility enabled the deduction of whether isolates resistant to CJLB-14 might have lacked flagella as group II phages target the

flagella upon infection. Results are shown in Table 7 below. There were no isolates that were non-motile and 4/16 were partially motile, leaving the rest as fully motile.

Table 7. Motility testing results. Isolates were labelled partially motile at measurements 1 cm ≤ x ≤ 2.5 cm and motile at measurements > 2.5 cm.

Caecal isolate	Radius (cm)	Motility result
P33/1	2.2	Partially motile
P33/2	2.8	Motile
P34/5	3.9	Motile
P34/6	3.9	Motile
P35/1	3.5	Motile
P35/2	3.5	Motile
P36/3	2.9	Motile
P36/4	1.1	Partially motile
P37/1	3.3	Motile
P37/2	2.5	Partially motile
P38/1	3.5	Motile
P38/2	3.7	Motile
P39/2	4	Motile
P39/9	4.1	Motile
P40/1	3	Motile
P40/2	2.2	Partially motile
C. jejuni PT14 control	4.4	Motile

3.2.5. Carrier state testing

Fourteen of the sixteen isolates were subcultured onto BA and used to make lawns. They were then tested to determine if they were carrier state phages. Lawns of both *C. jejuni* PT14 and *C. coli* NCTC 12668 were made. All the lawns of the resistant isolates, except P34/5, were complete and uniform, with no evidence of any plaques unless phage were applied, which indicated the isolates did not represent host bacteria in the carrier state (chronic-like phage infection). To test this hypothesis,

culture supernatants were collected from the resistant isolates and passed through a 0.2 μm filter before application of filtered suspensions onto lawns of *C. jejuni* PT14 and *C. coli* NCTC 12668. Plaques were observed for isolate P34/5 but no other (Table 8).

Table 8. Plaque formation from culture supernatants of the resistant isolates recovered from the caeca of phage-treated broiler chickens.

Caecal isolate	*C. jejuni* PT14 lawn	*C. coli* NCTC 12668 lawn
P33/1	-	-
P33/2	-	-
P34/5	+	-
P34/6	-	-
P35/1	-	-
P35/2	-	-
P36/3	-	-
P36/4	-	-
P37/1	-	-
P37/2	-	-
P38/1	-	-
P38/2	-	-
P39/2	-	-
P39/9	-	-
P40/1	-	-
P40/2	-	-

+, plaques and/or lysis; -, no plaques or lysis.

3.2.6. DNA sequencing

Seven of the sixteen resistant isolates were chosen for DNA isolation and sequencing, based on their categorisation from post-trial sensitivity testing, the motility test and carrier state test. The Illumina MiSeq system was used for DNA sequencing and the reads obtained were mapped to the genome of wild type *C. jejuni*

PT14. Table 9 below outlines common mutations found in all *C. jejuni* PT14 chicken isolates, reisolated from chickens, whether exhibiting wild-type characteristics or not. Table 11 displays the mutations found only in the resistant isolates from this study and Table 10 identifies which mutations from Table 11 were found in each of the resistant isolates that were sequenced.

Common mutations found in all *C. jejuni* PT14 chicken isolates, as seen in Table 9, include multiple single nucleotide variations in an L-lactate permease gene, an oxidoreductase subunit gene, the *flaA* flagellin gene and a *cheY* regulator gene. There was also an insertion event in an oligopeptide transporter gene, in which a polyA variant switched to phase off, and a deletion event in a gene encoding the putative periplasmic protein.

Table 10 shows that each isolate had various mutations associated with it that may have been the cause of their resistance towards the cocktail phages.

The mutations found only to be specific to *C. jejuni* chicken caeca resistant isolates, in Table 11, involve changes in the function and shape of structures, such as in motility, the CPS and cell wall, which are targeted by phage. Changes are also observed in signalling mechanisms, the aminoglycoside transferase system, SAM-dependent methyltransferases used in base modification, and in potassium transport systems, altering ion balance.

Table 9. Observed DNA mutations in the genome sequences of all *C. jejuni* PT14 isolates from chickens, sensitive or insensitive.

Reference position	Accession number	Frequency	Mutation type	Amino acid change	Gene product
88680	A911_00360	99.8	SNV	AFU42211.1:p.Ala169Pro	L-lactate permease
199939	A911_00990		Insertion	AFU42335.1:p.Asn301fs	polyA 8A variant phase off, OPT family oligopeptide transporter
500017	oorA, Gene: oorA	98.9	SNV	AFU42640.1:p.Asp221Gly	2-oxoglutarate-acceptor oxidoreductase subunit OorA
1267760 1267784	A911_06500	100	SNV	AFU43379.2:p.Lys262Gln AFU43379.2:p.Thr254Ala	FlaA flagellin
1277129	A911_06530	99.4	Deletion	AFU43731.1:p.Lys396fs	polyT 8T variant deacylase fusion to intergenic sequence. Putative periplasmic protein
1530548	A911_07740	100	SNV	AFU43610.1:p.Met95Val	Regulator consisting of CheY-like receiver domain and a winged-helix DNA-binding domain

Table 10. Sequenced caecal resistant isolates and the mutations associated with them, with reference to column 1 of Table 11: ‘Mutation reference no.’.

Caecal resistant isolate	**Associated mutations**
33/2	1, 2, 3, 4, 6
34/5	1, 3, 4, 5, 7, 8, 9
35/1	1, 3, 4, 9, 10, 11
35/2	1, 3, 4, 8, 9, 10, 11, 12
37/2	3, 4, 11, 13, 14
39/2	1, 3, 4, 5, 9, 11, 12, 15, 16, 17
40/1	3, 9, 11, 12, 14, 16, 18, 19, 20

Table 11. Observed nucleotide changes in the genome sequences specific to *C. jejuni* resistant chicken caeca isolates, compared to database wild type *C. jejuni* PT14, for which the DNA was isolated from a lab stock.

Mutation reference no.	Reference position	Accession number	Frequency	Mutation type	Amino acid change	Gene product	Resistance explanation
1	253740	A911_01335	100	SNV	AFU42394.1:p.Val298Ala	Rod shape-determining protein MreB- Actin-like ATPase involved in cell morphogenesis	Point mutation- single amino acid change causes a change in gene function and leads to a change in shape
2	577382	A911_03015	82.2	Deletion	AFU42719.1:p.Gly190fs	DUF2920 family protein hypothetical protein poly G 10G phase off	This protein has no known function but has the same effect as nos. 3 and 4
3	986299	A911_05085	99.3	Deletion	AFU43104.1:p.Phe358fs	COG0286 Type I restriction-modification system methyltransferase subunit	Change in modification enzyme, alters the base by adding a chemical. Perhaps turns off gene

							that codes for a phage target structure
4	1074552	A911_05520	82.0	Deletion	AFU43191.1:p.Gly114fs	COG0463 Glycosyltransferases involved in cell wall biogenesis, subject to phase variation of 11G (phase on) and 9G (phase off 75 %) at nucleotide 1,074,514. Alters GM1-like lipo-oligosaccharide	Wild type- 95 % had 11G phase on now 75 % have 9G phase off. Changes in cell wall lipopolysaccharide synthesis, CPS.
5	1193754	A911_06130	96.7	Insertion	AFU43309.1:p.Met373fs	COG0642 signal transduction histidine kinase	Gene is turned off due to frameshift, signalling mechanism affected.
6	1227308	A911_06290	80.3	Insertion	AFU43341.1:p.Ile60fs	Poly C 10C phase off variant normally 9C COG4310. Uncharacterized	Phase off now but was phase on in wild type isolates

						protein conserved in bacteria with an aminopeptidase-like domain	
7	1228778	A911_06295	82.5	Insertion	AFU43729.1:p.Val107fs	Phase variation: normally variable 9G to 11G at 1228738 Aminoglycosidase N3'-acetyltransferase	Changes in aminoglycosidase transferase system
8	1236346	A911_06345	85.4	Insertion	AFU43350.1:p.Ser197fs	DUF2920 protein with 9C but this has 10C phase off	This protein has no known function, but the mutation is common to all resistant isolates
9	1241712	A911_06370	83.2	Insertion	AFU43355.1:p.Ser197fs	polyC 10C variant phase off DUF2920 family protein. Phase variation of 9C (phase on) and 8C	This protein has no known function but leads to 8C phase off in 78 % and the mutation is common to 6/7 resistant isolates

						(phase off 78 %) at nucleotide 1,241,671	
10	1253136	A911_06440	76.9	Insertion	AFU43730.1:p.Tyr88fs	polyG 10G variant phase off normally 9G SAM dependent methyl transferase	Another SAM gene affected and turned off. Potential role in base modification
11	1263133	A911_06490	77.5	Insertion	AFU43377.1:p.Tyr60fs	COG2604 motility associated glycosyltransferase in Campy, normally shows phase variation of 9G (phase on) and 10G (phase off at 40 %) at nucleotide 1,263,092	Gene turned off. O-linked sugars on flagella altered so changes in motility-GII phages may be impaired in their recognition of glycosylated flagella
12	1353211	A911_06906	90.0	Deletion	AFU75866.1:p.Tyr135fs	polyG normally 8G with 55 % 10G but 30 % 11G variant places SAM-dependent	30 % are phase off from reading SAM-dependent methyltransferase,

						methyltransferase in frame	with a putative role in base modification
13	1354218	A911_06907	100	SNV	AFU75867.1:p.Val433Asp	Non-conservative point mutation in sugar transferase	Changes in CPS
14	1366679	A911_06918	100	Insertion	AFU75876.1:p.Tyr42fs	DUF2972 protein with 9C but this has 10C phase off	Uncharacterised protein
15	1369298	N/A	N/A	Deletion	N/A	Non-coding loss of T	N/A
16	1385873	A911_07000	81.2	Insertion	AFU43470.1:p.Tyr539fs	polyG 10G variant phase off normally 9G- sugar transferase.	Changes in CPS
17	1600146	A911_08110	99.2	Insertion	AFU43682.1:p.Ile286fs	polyA has 8A phase off normally 7A COG0475 Kef-type K+ transport systems, membrane components	Changes in ion balance

SNV = single nucleotide variation. CPS = Capsular polysaccharide. Fs = frameshift.

3.3. Discussion

Trial birds in group T2 (**2.7.**) were not infected with *Campylobacter jejuni* but were treated with CampyShield. During the trial, phage enumeration occurred for birds in all groups, T1-T5. As expected, no phages were recovered from birds in groups T1 and T3 as they were not treated with phage. Phages were not recovered from the caecal contents of T2 birds. This can be explained by the absence of *Campylobacter* colonisation, as phage recovery appears dependent on the presence of their host in the environment of the gut. Phages are obligate organisms that require a specific bacterial host to multiply rapidly (Stone *et al*., 2019), and by inference replication, *in situ* is necessary for the phages to persist to be recovered rather than intestinal transit over 24 h after oral gavage and necroscopy. Kittler *et al*., (2013), outlined that in commercial broiler houses, the presence of susceptible host bacteria is vital for the application of phages and the subsequent reduction of *Campylobacter* counts. The phages comprised in CampyShield are *Campylobacter*-specific phages and therefore, require *Campylobacter* spp. such as *C. jejuni* or *C. coli* to survive and replicate. Consequently, the phages given to the T2 birds were unable to proliferate and multiply in the absence of *Campylobacter* with any remaining phage falling below the minimum limit of detection (> 200 PFU g^{-1}).

All *Campylobacter* isolates were tested for phage resistance before the trial was conducted and they were dosed at specific titres; no resistance was detected. However, the emergence of phage-resistant campylobacters post-treatment was observed in this study as previously reported (Richards *et al*., 2019). The results obtained in Table 6 indicate that monophages CJLB-5 and CJLB-10 and the combination in CampyShield were ineffective against these campylobacters post treatment. Based on these results, the sensitivity of isolate P39/2 to CJLB-5 and CJLB-10 (group III phages) and resistance to CJLB-14 (group II) indicate that this isolate may have become resistant through flagella modification or loss; group II phages contact the host by targeting the flagella (Olson *et al*., 2022). However, the results from the motility test and DNA sequencing indicate that this isolate was motile; a radius of 4 cm was observed. Therefore, isolate P39/2 was not lacking flagella function, unlike resistant variants to group II phages that are reported as non-motile (Sørensen *et al*., 2015). On the other hand, the isolates resistant to CJLB-5

and CJLB-10 phages but sensitive to CJLB-14 may have lost or possessed modified surface molecules, such as on the capsular polysaccharide; CJLB-5 and -10 are group III phages which use capsule polysaccharide to attach to their hosts (Olson *et al*., 2022). DNA sequencing was used to confirm possible resistance mechanisms.

Further post-trial sensitivity testing highlighted the success of CP30A against these resistant T5 isolates. While all isolates were resistant to at least two of the CampyShield phages, or in several cases, all of the phages, every isolate proved to be sensitive to CP30A. This could be explained by the fact that T5 isolates were not exposed to CP30A throughout the experiment, as they were treated with CampyShield. As mentioned, resistance occurs through rapid mutations to confer mechanisms that aid their survival in the conditions they reside. Therefore, exposure of the T5 isolates to the CampyShield phages throughout the trial would allow the campylobacters to evolve resistance mechanisms to them. However, upon observation of T4 sensitivity results to CP30A and T5 sensitivity results to CampyShield, it is clear that CP30A is more effective. Firstly, the trial observed a significant difference between the average reduction of *Campylobacter* counts per gram of caecal contents by CampyShield and CP30A. CampyShield produced a 1.45 $\log_{10}$ CFU g^{-1} reduction in the T5 group ($p_{adj} = 0.005$), while CP30A produced a 2.94 $\log_{10}$ CFU g^{-1} in the T4 group ($p_{adj} = 0.00001$). This indicates that already in the caeca, CP30A was much more effective at reducing *Campylobacter* counts, by almost 1.5 $\log_{10}$ CFUg^{-1}. Consequently, replacing either CJLB-5 or CJLB-10 with CP30A in the cocktail, may offer much better results in future trials of *Campylobacter* reduction in broiler caeca, as these were the two monophages that experienced the most resistance, as shown in Table 6.

CP30A has been successful in several studies when used to reduce *Campylobacter* counts in poultry production. Richards *et al*., (2019), observed a 2.4 $\log_{10}$ CFU g^{-1} reduction of *C. jejuni* counts in the caeca of broilers using a cocktail that contained CP30A and a group II phage. In this trial, Richards *et al*., (2019), observed a resistance level of 4.5 % to CP30A post treatment. We observed a 24 % resistance level in the T4 isolates to CP30A, which although, is higher than observations of Richards *et al*., (2019), it was still much lower than the resistance level observed for CampyShield in T5 isolates, at 65 %. A mutation frequency of 65 % is high

compared to results from other studies. Al Hakeem *et al*., (2022), compared phage resistance among *C. jejuni* caecal isolates of broilers and concluded that the single phage resulted in a mutation frequency of 43 %, whereas the cocktail observed that at 24 %. Furthermore, Olson *et al* (2022) recorded the highest mutation frequency of *C. jejuni* in broilers at 43 %. On the other hand, Nowaczek *et al*., (2019), reported that 67 % of *Campylobacter* isolated from poultry were resistant to all phages tested. The implications of reusing the phages if resistance has reached 65% of *Campylobacter* isolates after a production cycle is that it may not work if the isolates can continue to colonise chickens. For this reason, it is recommended to use the phage before clearing the barn for processing, to prevent the dissemination of resistant types. However, phage resistant mutants are often impaired in their ability to colonise and compete with wild type bacteria. For example, impaired motility can prevent phage infection but also prevents chicken colonisations, and therefore, it is likely that phages will encounter them as they are not fit for survival.

Carrier state testing was also used in an attempt to discover the resistance mechanism of the isolates. As mentioned, carrier state phages are mixtures of bacteria and phage that remain associated with each other in a stable equilibrium. Furthermore, carrier state cultures can either contain sensitive bacteria that support phage replication or resistant bacteria that avoid infection but can continue to act as founders of the sensitive population (Siringan, *et al*., 2014). This allows isolates to survive in bacteriophage-infested environments as they develop certain mechanisms to help them adapt. For example, carrier state colonies of *C. jejuni* PT14 can tolerate the presence of oxygen at a higher capacity, in conditions where nutrients are limited; this may have been an evolutionary response to pressures in the extra-intestinal environment (Hooton, *et al*., 2016). They may also be lacking motility due to the presence of an *flhF* mutant. FlhF is a key protein needed to finalise flagellar synthesis and its deletion results in the absence of flagella in *C. jejuni* so they become non-motile (Li *et al*., 2020). The results obtained from carrier state testing in this study indicated that only one isolate, P34/5, had potentially entered the carrier state. This isolate lysed the *C. jejuni* PT14 lawn, indicating the presence of phage, and therefore, a carrier state isolate, which could be the reason for resistance by this isolate. Resistance of P34/5 to CJLB-14 and the positive carrier state result could suggest the presence of an *flhF* mutant. However, results from the motility test and

from DNA sequencing indicated this isolate was motile so P34/5 possessed an alternative resistance mechanism. Although carrier state campylobacters may become resistant to phages, Brathwaite *et al*., (2015), stated that *C. jejuni* carrier state cultures are unable to efficiently colonise chickens and that carrier state phage were able to replicate and reduce the *Campylobacter* count of pre-colonised chickens. This indicates that *C. jejuni* carrier state cultures lack the ability to successfully colonise their hosts so although they are not eliminated, they are less of a threat, if one at all.

DNA sequencing was carried out for several resistant isolates to compare the sequences of resistant and sensitive strains, identifying phase variation involving nucleotide changes and mutations that may confer resistance. Phase variation is the inheritable, interchangeable and high frequency switching in gene expression, which is regulated by various mechanisms and is frequently observed in *C. jejuni* to adapt to different environments and host niches (Vasu and Nagaraja, 2013; Wanford *et al*., 2018). For example, the passage of *C. jejuni* through chicks may result in phase variation that is heavily associated with colonisation and disease (Truccollo *et al*., 2021). Many *Campylobacter*-specific phages depend upon phase-variable surface structures to successfully infect the bacteria (Aidley *et al*., 2017) and therefore, phase-variable genes modulate the expression of these surface molecules, influencing phage binding and potentially conferring phage resistance.

Table 9 shows the mutations observed in the genome sequence of all *C. jejuni* isolates recovered from chicken caeca, phage resistant or not. These are mutations that have been acquired in response to colonisation by *C. jejuni* PT14, compared to the laboratory propagated wild type strain. Selection will have a role in this process but the populations that encounter the environmental selection may be limited due to the bottleneck effect. Bacteria have frequently been observed to undergo population bottlenecks during transmission between hosts and during migration between host environments (Wanford *et al*., 2018). A non-selective bottleneck effect results in only a proportion of the bacterial population being carried over to the new niche and leads to dramatic reductions in the genetic diversity of the bacterial population (Croix *et al.*, 2020); these have previously been observed in broiler colonisation experiments with *C. jejuni* (Cayrou *et al*., 2021). In this study, the mutations outlined

in Table 9 identify the nucleotide changes, common among all *C. jejuni* isolates, that have arisen from a combination of chance due to the bottleneck effect during colonisation of the trial birds and selection from a subset of phasotypes upon populating new intestinal niches. Of note are the changes in *flaA* – the major flagellin associated gene responsible for flagellar structure and motility. These are 4 nucleotide changes, 2 of which are silent and 2 that result in the point mutations T254A and K262Q. Database searches (BLAST-P) of the FlaA variant protein sequence revealed these amino acids to be common in *C. jejuni* poultry isolates and may confer fitness in vivo.

Table 11 identifies mutations that are restricted to the phage resistant caecal isolates. The nucleotide changes observed indicate there are common SNVs, deletions and insertions of nucleotides in the coding regions of the genomes of campylobacters recovered from different birds. SNVs create point mutations, whereas deletions and insertions lead to frameshift mutations and frameshift mutations result in genes switching from an ON phase to an OFF phase (Wanford *et al*., 2018). Mutation 1 is a point mutation leading to the substitution A53V in the MreB protein that is responsible for the maintenance of a rod-shaped cell (Figge *et al*., 2004), where *mreB* mutations show changes in cell morphology. In *C. jejuni*, the reduced expression of *mreB* has been observed to result in a spherical phenotype and variation in the gene has been linked with adaptation to new conditions and localisation of virulence proteins in the cell (Wheeler *et al*., 2019). This mutation, therefore, may have enabled the isolates that carried mutation 1 to evade infection by CampyShield phages due to changes in cell shape. Van Teeseling *et al*., (2017), stated that shape dictates the interactions between a bacterial cell and its environment, such as motility, host colonisation (including pathogenesis) and resistance and therefore, cell morphology is a major evolutionary and adaptive process that plays a role in bacterial environmental presence.

Phase variable CPS structures are a common reason for resistance to bacteriophages by *C. jejuni*, particularly to group III phages. This includes the polysaccharide biosynthesis regions that exhibit variable genomic regions among *C. jejuni* (Parker *et al*., 2008). Changes in these structures emerge upon constant exposure to phages in chicken caeca and this exposure results in continuous phage–host co-evolution

(Ushanov *et al*., 2020). This highlights the likelihood of *C. jejuni* developing these mutations in groups T4 and T5 as they were constantly exposed to phages. Isolate 33/2 experienced two types of mutations in CPS biosynthesis genes (mutations 4 and 13 in Table 11). Mutation no. 4 involved the loss of two G residues in a polynucleotide tract that switched the dominant phasotype from 95 % phase ON to 75 % phase OFF, altering the expression of the glycosyltransferase function, involved in cell wall biogenesis. Mutation no. 13 was a point mutation in a sugar transferase, which aids in the synthesis of the CPS in bacteria (Miyake and Iijima, 2004). Therefore, these mutations may result in changes to surface structures, enabling isolates to avoid recognition by bacteriophages. Resistance of isolate 33/2 to CampyShield, CJLB-5 and CJLB-10 may be explained by these mutations as group III phages target the CPS and the structures on the surface that may include lipopolysaccharides.

Likewise, all isolates possessing mutation 16 in addition to 4 and 13 may be similarly resistant to those phages in this way, as mutation 16 resulted in a framseshift mutation of a sugar transferase gene, leading to genes being turned to phase OFF. These findings are supported by various studies, including the one above by Parker *et al*., (2008), which observed several mechanisms that lead to changes in lipooligosaccharide structures among *C. jejuni*. Gene inactivation, including missense mutations, phase variable and non-phase variable inactivation through deletion or insertion of bases was shown to be a principal mechanism in altering the lipooligosaccharide structures. Gilbert *et al*., (2001), also identified several mechanisms that allow *C. jejuni* to vary the outer core of the lipooligosaccharide structure. This included phase variation, gene inactivation by the insertion or deletion of a base and single mutations that lead to the inactivation of a glycosyltransferase. Mutation 4 also alters the GM1-like lipo-oligosaccharide, which is associated with the ganglioside GM1-like lipo-oligosaccharide (Linton *et al*., 2002). Ganglioside mimicry by *C. jejuni* lipo-oligosaccharide plays a key role in the development of Guillain–Barré and Miller–Fisher syndrome after *C. jejuni* infection.

Mutations 10 and 12 observed changes to S-adenosylmethionine (SAM)-dependent methyltransferases which resulted in genes being turned to phase OFF, (30 % in mutation 12), affecting a modified base machinery protein. SAM-dependent

methyltransferases are involved in the modification of lipids, proteins and nucleic acids and in the biosynthesis of small molecule metabolites (Sun *et al*., 2021). Vesel *et al*., (2023), identified that DNA modifications in *C. jejuni* affected transformability and promoted DNA uptake. Type I restriction and modification systems involve enzymes with several functions that catalyse restriction and modification and can be linked to SAM-dependent methyltransferases. Restriction-modification systems are reported to be common anti-phage defences that detect and attack foreign DNA in bacteria. Type I enzymes bind to their target sequence and their role as an endonuclease or methyltransferase is determined by the methylation state of the target sequence (Murray, 2000). Restriction nucleases cleave foreign DNA at specific sites and methyltransferases modify sequences in the bacteria's DNA to avoid self-destruction (Arias *et al*., 2022). Mutation 3 resulted in a frameshift of a modification enzyme: a methyltransferase subunit, of a Type I restriction-modification system. Isolate 39/2 was the only isolate to possess this mutation. This isolate was also the only one to exhibit sensitivity to phages CJLB-5 and CJLB-10 (Table 6) which could be explained if the isolate was impaired in its ability to detect and attack the foreign phage DNA, leading to phage infection. Alternatively, this mutation could be responsible for the isolate's resistance towards CJLB-14 if the phage exhibited dependence on the modification, as it has been reported by Vesel *et al*., (2023), that a specific methylation motif, the result of a *Campylobacter*-specific orphan methylase, promoted DNA uptake in *C. jejuni*.

Mutation 11 led to a gene associated with glycosyltransferase activity involved in O-linked protein glycosylation being switched to 40 % phase OFF. FlaA flagellin is post-translationally modified by O-linked glycosylation with the attachment of pseudaminic acid and sometimes legionaminic acid (Thibault *et al*., 2001; Zebian *et al*., 2016). It is notable that one of the mutations encountered in the *flaA* gene would substitute the potential attachment site of threonine at 254 with a conservative but non-modifiable residue alanine. Only one isolate: 40/1, exhibited mutation 11. This isolate was motile, producing a radius of 3 cm upon motility testing. Upon sensitivity testing to phages, the isolate exhibited resistance to CampyShield, CJLB-5 and CJLB-10 but was sensitive to CJLB-14. Based on previous discussions, mutations in the flagella are usually related to *C. jejuni* resistance towards group II phages. However, a conserved group III phage protein, FlaGrab, is reported to bind 7-

acetamidino-pseudaminic acid decorating FlaA flagellin, which can partially inhibit cell growth (Sacher *et al*., 2020). At least partial avoidance of the binding of FlaGrab in a phage rich environment would alleviate the growth handicap.

Lis and Connerton (2016), identified that group III phages can have a modified function with respect to motility. A group III phage, CP_F1, produced clear lysis on a *C. jejuni flaB* mutant and complementation of the *flaB* mutant partly restored opaque lysis, while on the wild type strain, CP_F1 restored opaque lysis caused by regrowth of a *Campylobacter* sub population that avoided phage infection. On *flaA* and *flaAB* lawns, opaque plaques were observed, while on the *kpsM* mutant, there was no plaque formation. These results indicate that knocking out the *flaA* gene in *C. jejuni* PT14, resulting in a completely non-motile isolate, leads to impaired infection by group III phages as mutants exhibited reduced efficiency of plating. Therefore, mutation 11 in isolate 40/1, causing changes in motility may be the cause of resistance to CJLB-5 and CJLB-10 phages. Isolate 40/1 was also the only isolate that held mutations 14 and 17. The impact of mutation 14 remains unknown as the DUF2972 protein is uncharacterised; investigations into this protein would require further studies so appropriate conclusions can be drawn. However, mutation 17 involves phase variation in K+ transport systems and membrane components. This could confer a phage resistance mechanism involving potassium (K+) ion influx modulation and enhanced biofilm formation, a newly discovered phage resistance mechanism (Tzipilevich and Benfey, 2021).

Mutation 5 observed phase variation in a signal transduction kinase, where the gene was turned off due a frameshift mutation. These kinases are important for bacterial communication as the histidine kinase senses a specific stimulus in the environment, initiating the response regulator to moderate the cellular response through differential expression of target genes (Mascher *et al*., 2006).

Lastly, all resistant isolates possessed mutations in the DUF2920 protein (mutations 2, 8 and 9) but as yet, this protein has no known function. It is suspected that phase variation in this protein might have a role in immune evasion or result in changes to the CPS. This finding is significant in that these mutations may be the cause of resistance to the CampyShield phages by these isolates as they are present in each.

However, future studies involving further investigations into this protein are required to draw suitable conclusions.

Results from the trial initially proved that CP30A was a more effective phage in reducing *Campylobacter* counts in broiler chicken caeca. In addition, CampyShield failed to prove effective against the campylobacters used in the trial as the T5 caecal isolates displayed resistance post treatment. Al Hakeem *et al*., (2022) has outlined how the development of phage resistance might limit the use of *Campylobacter*-phages in the biocontrol of *Campylobacter*. Furthermore, only 24 % of group T4 isolates exhibited resistance to CP30A post treatment, whereas, 65 % of group T5 isolates were resistant to CampyShield post treatment. Perhaps substituting one of the monophages: CJLB-5 or -10 with CP30A in the CampyShield cocktail would offer a higher success rate against these campylobacters in the caeca of broilers. The DNA sequencing results indicate that there was a high degree of phase variation that led to resistance among the isolates. Notable changes were observed in mutations of the CPS and cell-wall, including glycosyltransferases, lipo-oligosaccharides and sugar transferases, perhaps being the cause of resistance to the group III phages. Phase variation in the DUF2920 protein was also significant, being present in all isolates but not having a known function. Therefore, further studies would be necessary to investigate the function of this protein. Further studies would also be necessary to investigate phages that successfully target these isolates for future therapeutic purposes. Phages in contention must produce a lower mutation frequency post-treatment and be responsible for a large reduction in *Campylobacter* counts, like that of CP30A.

Chapter 4- Investigating the Liver

4.1. Introduction

Colonisation by *Campylobacter* of the broiler chicken gut can extend to the liver, spleen, deep muscle, thymus and bursa of Fabricius (Patuzzi *et al*., 2021). Chicken liver is in close proximity to the intestinal tract and has been the vehicle of many food-borne infections by *Campylobacter* and *Salmonella,* as they spread through the biliary, lymphatic or vascular systems (Blanco-Picazo *et al*., 2022). Chicken liver pâté is a popular delicacy in Northern and Eastern Europe and is a food commonly associated with campylobacteriosis, indeed like many other chicken liver-based dishes. The prevalence of *Campylobacter* in chicken liver varies by country. An Australian study investigated its prevalence on the external surface and internal part of chicken livers sold in New South Wales. A sample of 255 livers from 51 batches were tested to reveal a *Campylobacter* prevalence of 96 %. Similarly, a study from New Zealand found that 90 % of 30 livers tested positive for internalised *Campylobacter* and a study from Scotland recorded that 81 % of a ~382 g sample of retail livers was externally positive (New South Wales Government Food Authority, 2018). For this reason, whether the application of bacteriophages can reduce these incidences represents an important research question. Investigations have focussed on evidence for the translocation of *Campylobacter* phages from the broiler chicken intestines to the liver and if so, how might this be achieved.

Blanco-Picazo *et al*., (2022), investigated the presence of phages in chicken livers and discovered that phages can translocate from the gut to the liver. This chapter reports investigations into the presence of *Campylobacter* in the livers of *Campylobacter*-colonised trial birds (**2.6.**) and whether CampyShield and CP30A were able to translocate from the gut into the liver post application. Livers were collected at necroscopy and stored at -80 °C, from which they were thawed and dissected to produce liver homogenates. Enrichment and increased sensitivity techniques were required to reach detection limits of *Campylobacter* counts and phage titres. A summary of the trial study design is reproduced in Table 12 and can also be found in section **2.6.2**.

Table 12. Study design of the broiler chicken trial.

Treatment group (pen numbers)	Treatment Description	Dose of phage	Days receiving phage
T1 (P1-8)	No Challenge No Phage	-	-
T2 (P9-16)	No Challenge CampyShield	10^9 PFU	39 & 40
T3 (P17-P24)	Challenge No phage	-	-
T4 (P25-32)	Challenge CP30A phage	10^9 PFU	39 & 40
T5 (P33-40)	Challenge CampyShield	10^9 PFU	39 & 40

4.2. Results

4.2.1. Isolating *Campylobacter* from broiler chicken livers (Trial groups T1-5)

Isolation of *Campylobacter* was attempted from all livers (the control group T1 birds were negative as expected) but several attempts were made from group T2 (non-colonised but with phage application), likewise with groups T3-5- a particular focal point, as they were challenged. However, despite using direct selective plating on mCCDA and enrichment to increase the sensitivity, the results were negative for all birds tested.

4.2.2. Isolating *Campylobacter* phage from broiler chicken livers (Trial groups T1-5)

Phage isolation was carried out on livers from birds from all groups (pens 1-40). Group T1 livers offered negative results, as expected. Direct application of homogenate dilutions onto bacterial lawns, enrichment and increased sensitivity methods were employed for groups T2-T5. Negative results were observed for all livers subjected to the direct method.

4.2.2.1. Enrichment technique

This technique used an enrichment broth containing aliquots of liver homogenates with *C. jejuni* PT14 and livers from birds in groups T2-5. Results are displayed in Table 13 below. The table displays that only positive results were obtained from two birds, in group T5 (P35 and P38).

Table 13. Results from the enrichment technique used to isolate phage from livers in groups T2-T5. Positive and negative results were determined based on the presence or absence of plaques/lysis. P = pen. +, plaques and/or lysis; -, no plaques or lysis.

Liver sample (bird number)	Phage presence	Group
P12/1	-	T2
P13/1	-	
P14/1	-	
P15/1	-	
P16/1	-	
P17/1	-	T3
P18/1	-	
P19/1	-	
P20/1	-	
P21/1	-	
P22/1	-	
P23/1	-	
P24/1	-	
P25/1	-	T4
P26/1	-	
P27/1	-	
P28/1	-	
P29/1	-	
P30/1	-	
P31/1	-	
P32/1	-	
P33/1	-	T5
P34/1	-	
P35/1	+	
P36/1	-	
P37/1	-	
P38/1	+	
P39/1	-	
P40/1	-	

4.2.2.2. Increased sensitivity technique round 1

This technique used whole liver homogenates in MRD added to an equal volume of double strength enrichment broth with *C. jejuni* PT14 and livers from birds in groups T2, T4 and T5. This aimed to enrich the phages further with their host, as results were mainly negative after using the first enrichment technique. The results are displayed in Table 14 below. The table displays that only positive results were obtained from a few birds in groups T4 and T5.

Table 14. Results from round 1 of the increased sensitivity technique used to isolate phage from livers of birds in groups T2, T4 and T5. Positive and negative results were determined based on the presence or absence of plaques/lysis. P = pen.

Liver sample (bird number)	Phage presence	Group
P12/1	-	T2
P13/1	-	
P14/1	-	
P15/1	-	
P16/1	-	
P25/1	+	T4
P26/1	-	
P27/1	-	
P28/1	+	
P29/1	-	
P30/1	+	
P31/1	-	
P32/1	-	
P33/1	-	T5
P34/1	-	
P35/1	+	
P36/1	-	
P37/1	-	
P38/1	+	
P39/1	-	
P40/1	+	

+, plaques and/or lysis; -, no plaques or lysis.

4.2.2.3. Increased sensitivity technique round 2

The same technique was used as in round 1 but this round used livers excised from different birds in each pen of groups 4 and 5. Lawns for spotting used *C. coli* NCTC 12668 (for group T5 only) as well as *C. jejuni* PT14. Results are displayed in Table

15 below. The table displays that positive results were obtained from almost all liver samples in both groups on the *C. jejuni* PT14 lawns.

Table 15. Results from round 2 of the increased sensitivity technique used to isolate phage from livers of different birds in groups T4 and T5. Results were recorded differently compared to round 1; birds were labelled with ≤ 10 plaques if there were also no plaques and with ≥ 100 plaques if there was lysis as well as plaques. P (liver sample) = pen.

Liver sample (bird number)	*C. coli* NCTC 12668	*C.jejuni* PT14	Group
P25/2	/	10 < x < 100 P	T4
P26/2	/	10 < x < 100 P	
P27/2	/	10 < x < 100 P	
P28/2	/	10 < x < 100 P	
P29/2	/	10 < x < 100 P	
P30/2	/	≥ 100 P	
P31/2	/	10 < x < 100 P	
P32/2	/	≤ 10 P	
P33/2	≤ 10 P	10 < x < 100 P	T5
P34/2	≤ 10 P	≤ 10 P	
P35/2	≤ 10 P	10 < x < 100 P	
P36/2	≤ 10 P	≤ 10 P	
P37/2	≤ 10 P	10 < x < 100 P	
P38/2	≤ 10 P	10 < x < 100 P	
P39/2	≤ 10 P	10 < x < 100 P	
P40/2	≤ 10 P	≥ 100 P	

/, did not carry out; P, plaques.

4.2.3. Propagation of group T4 and T5 liver plaques

The plaques isolated from the lawns after round 2 of the increased sensitivity method were picked and propagated onto *C. jejuni* strains; NCTC 12662 and PT14. Results are shown in Table 16 below. Negative results were obtained from *C. jejuni* NCTC

12662 lawns and positive results were obtained from all *C. jejuni* PT14 lawns. P32 and P36 samples were excluded due to insufficient results.

Table 16. Results from the propagation of T4 and T5 liver plaques after increased sensitivity round 2. Results were labelled positive in the presence of lysis/plaques and negative in the absence of lysis/plaques.

Liver sample (bird number)	*C. jejuni* NCTC 12662	*C. jejuni* PT14	Group
P25/2	-	+	T4
P26/2	-	+	
P27/2	-	+	
P28/2	-	+	
P29/2	-	++	
P30/2	-	++	
P31/2	-	++	
P33/2	-	++	T5
P34/2	-	+	
P35/2	-	+	
P37/2	-	+	
P38/2	-	+	
P39/2	-	+	
P40/2	-	+	

+, complete cell lysis; ++; complete cell lysis and > 50 plaques; -, negative (no plaques).

4.2.4. Spike experiment of group T3 livers

The 'Spike' experiment was conducted on three livers from birds P22, P23 and P24. The experiment involved ascertaining the limit of detection for these livers if phage were to be present. Direct and enriched samples were used. Plaques and lysis were seen on direct plates at the 10^{-3} dilution and on enriched plates at the 10^{-5} dilution. Therefore, the limit of detection was ≥ 2000 PFU for direct samples and ≥ 20 PFU for enriched samples.

4.2.5. Obtaining the phage limit of detection for group T3 and T4 livers

Liver samples from groups T3 and T4 were subjected to titrations to discover their phage detection limit. Direct and enriched homogenates were used and subjected to a 10-fold dilution series for spotting onto *C. jejuni* PT14 lawns. Detection limits are shown in Table 17 below; results show that only three samples produced plaques.

Table 17. Results from detection limit investigations using T3 and T4 direct and enriched liver samples. Positive results indicate the presence of phage and negative results indicate the absence of phage. Plaques were counted at dilutions of 10^{-3} for direct samples and at 10^{-5} for enriched samples.

Liver sample (pen number)	Direct	Enriched	Approx. PFU g^{-1}	Group
P17/1	-	-	$< 2 \times 10^1$	T3
P18/1	-	-	$< 2 \times 10^1$	
P19/1	-	-	$< 2 \times 10^1$	
P20/1	-	-	$< 2 \times 10^1$	
P21/1	-	-	$< 2 \times 10^1$	
P22/1	-	-	$< 2 \times 10^1$	
P23/1	-	-	$< 2 \times 10^1$	
P24/1	-	-	$< 2 \times 10^1$	
P25/1	-	-	$< 2 \times 10^1$	T4
P26/1	-	-	$< 2 \times 10^1$	
P27/1	-	-	$< 2 \times 10^1$	
P28/1	-	+	$2 \times 10^1 < x < 2x\ 10^4$	
P29/1	-	+	$2 \times 10^1 < x < 2x\ 10^4$	
P30/1	-	+	$2 \times 10^1 < x < 2x\ 10^4$	
P31/1	-	-	$< 2 \times 10^1$	
P32/1	-	-	$< 2 \times 10^1$	

+, positive/plaques; -, negative/no plaques.

4.2.6. Determining if CJLB-14 can get into the liver

Group T2 and T5 liver homogenates and *C. coli* 12668 suspensions were used to test whether the CJLB-14 monophage (group II) in the CampyShield cocktail translocated from the gut into the liver. All livers from birds P12-16 and from P33-40 yielded negative results, as did the control, indicating that CJLB-14 failed to translocate from the gut into the liver.

4.3. Discussion

Negative results obtained from *Campylobacter* isolation of livers in groups T3-T5 indicate that *Campylobacter* was unable to translocate from the caeca to the liver in this experiment. This may be explained through the infection and subsequent killing of *Campylobacter* by phage, in groups T4 and T5, during translocation to the liver and during the experimental techniques such as stomaching and enrichment. For group T3, absence of campylobacters may be due to their inability to translocate through the gut lining to the liver. This finding appears contrary to the observations of Firelyanti *et al*., (2016), that campylobacters could be recovered from the internal tissues of retail chicken livers and experimentally colonised birds. However, Firelyanti *et al*., (2016) noted that although campylobacters could be recovered from chickens colonised by liver *C. jejuni* isolates, they failed to recover campylobacters, from the extra-intestinal organs, of the control *C. jejuni* strains HPC5 or 81-176 routinely propagated in the laboratory. Bacteria has been observed to cross the intestinal barrier of animals and humans and campylobacters have been identified to do this in *in vitro* studies; they have been found to translocate using the transcellular passage through enterocytes or paracellular routes through tight junctions (Louwen *et al*., 2012). In addition, Backert *et al*., (2013), indicated that *C. jejuni* was able to invade underlying tissues, enter the bloodstream and possibly reach organs such as the liver. Truccollo *et al*., (2021), found that *Campylobacter* transmission in broilers depends on the ability of the bacteria to withstand several stresses such as oxidative, osmotic, desiccation and thermal. They are also required to compete with natural microbiota and evade the host immune responses. Perhaps the campylobacters used to infect the broilers in the trial were unable to tolerate these selection pressures and therefore, failed to translocate to the liver.

Upon attempted phage isolation from groups T2, T3, T4 and T5 livers, phages were only isolated from groups T4 and T5 (T3 were negative as expected; they were not

administered with phage). This indicated they were able to translocate from the gut into the liver. We are certain that these phages were not present due to contamination as careful and good aseptic techniques were used, control tests indicated the absence of phage and test plates only showed plaques where they were dispensed onto the plate. In addition, postmortem handling and necroscopy of the birds and organs was performed under sterile conditions and using appropriate techniques; organs were excised prior to breaking the integrity of the intestines. Organs were also stored separately and under sterile conditions in the laboratory. Moreover, the livers of the birds that were not administered phage remained free of phage (groups T1 and T3) and notably, the phage only group (T2) that could have become contaminated were also devoid of phage in the liver.

The lack of phages present in group T2 livers along with the presence of phages in group T4 and T5 livers, indicated that the phages needed the campylobacters to be present in the gut, for replication and to be carried to the gut lining and therefore, to translocate into the liver. It is suggested that the phages in groups T4 and T5 birds were carried by campylobacters to the hepatic portal vein (HPV) of the gastrointestinal tract and translocated through the blood stream to the liver independently of their *Campylobacter* host. This occurs as the bacteria in the caeca replicate near the epithelium due to the oxygen supply from the blood. Van Deun *et al*., (2008), gathered data supporting this hypothesis and suggested that *C. jejuni* was able to avoid being expelled from the chicken gut through rapid multiplication in the mucus and that *C. jejuni* closely interacts with the epithelial lining of the caecum, which leads to translocation to the liver. Although no campylobacters were found in livers in the current study, the findings in the study by Van Deun *et al*., (2008), give evidence that the phages were able to translocate in this manner independently of their host. Blanco-Picazo *et al*., (2022), highlighted that other sources have shown phage translocation from the intestinal tract to the bloodstream and concluded that the findings in their study indicated phage translocation from the intestinal tract to the liver.

The results obtained from increased sensitivity testing round 2 on the *C. coli* NCTC 12668 lawn indicated that the host range of the CampyShield cocktail does not extend to *C. coli,* despite containing CJLB-14, a group II phage. This proves

disadvantageous for the efficacy of the cocktail in broiler *Campylobacter* treatment as *C. coli* is the second most common *Campylobacter* species to be found in chickens and a broad host range is extremely important when considering the efficacy of therapeutic phages. Testing of CP30A (group T4 samples) on the *C. coli* NCTC 12668 lawns was not necessary as the host range of this phage only extends to *C. jejuni* strains (Olson *et al*., 2022). Results in Table 15 alongside those in Table 16 indicate that CP30A and CampyShield are extremely effective phages against *C. jejuni* PT14. On the other hand, these phages proved to be ineffective against *C. jejuni* NCTC 12662. The efficacy of these phages towards *C. jejuni* PT14 is expected as this strain is referred to as the universal *Campylobacter* bacteriophage host strain so has been widely used to isolate *C. jejuni* phages due to its high phage sensitivity (Sørensen *et al*., 2015). It is surprising, however, that none of the phages from the livers in groups 4 or 5 could infect *C. jejuni* NCTC 12662 as this is also a universal *Campylobacter* bacteriophage host strain. Perhaps this strain does not have as wide a host range as the label implies or resistance has arisen due to changes in the strain itself; constant and routine subculturing may contribute to genetic variation. Campylobacters are said to be highly genetically diverse due to frequent recombination, which can confer virulence and survival mechanisms to withstand environmental pressures (Truccollo *et al*., 2021). However, this hypothesis cannot be confirmed without PCR amplification and genome sequencing to identify potential genetic changes, which could be carried out in further studies.

Table 17 produced expected negative results for group T3 liver samples and some positive results for group T4 samples. The direct technique allowed only for the detection of phages at titres of 2000 PFU or above and the enrichment technique allowed only for the detection of phages at counts of 20 PFU or above.

The ‘Spike’ experiment was conducted to discover the limit of detection of group T3 livers if phages were to be present. This was conducted through adding CP30A phage and *C. jejuni* PT14, as food for the phage, to liver homogenates, to see how much phage could be isolated. The detection limit of phages at $\geq 10^5$ PFU ml^{-1} for direct samples and $\geq 10^3$ PFU ml^{-1} for enriched samples is supported by the results obtained in Table 17.

CJLB-14, as a group II phage, replicates well on *C. coli.* Group II phages infect both *C. coli* and *C. jejuni*, while group III are largely restricted to *C. jejuni* (Steffan *et al*., 2022). CJLB-14 failed to replicate well on *C. jejuni* PT14 but it did replicate well on *C. coli* NCTC 12668 so this strain was used to determine whether CJLB-14 translocated to the liver as CJLB-10, a group III phage, achieved this (Table 19, next chapter). Using both *C. jejuni* PT14 and *C. coli* NCTC 12668 covers the host ranges of all three phages to determine which of the monophages were able to translocate into the liver. Negative results indicated that there was no CJLB-14 present in the livers so only CJLB-10 translocated. These findings are supported by the Firlieyanti *et al*., (2016) study, which stated that none of the phages isolated from chicken liver infected *C. coli* isolates, implying they were group III. In addition, upon PFGE analysis, by Firlieyanti *et al*., (2016), of the bacteriophage genomic DNA, the three phages isolated from chicken liver revealed genome sizes of ~140 kb, typical of group III phages and PCR amplification of the DNAs with group III-specific primers confirmed they were group III phages.

Based on the above findings, *Campylobacter* isolates were unable to translocate from the caeca into the liver, perhaps due to their inability to withstand the environmental pressures of the broiler intestines and blood vessels. The phages however, were able to translocate to the liver, perhaps with the help of the *Campylobacter* isolates, transporting them to the gut lining. Table 15 indicates that both CP30A and CampyShield were able to translocate to the liver however, (Table 19, next chapter) and results from (**4.2.6.**) indicate that only CJLB-10 of the cocktail was present in the livers. In terms of the efficacy of the cocktail for therapeutic use in broiler livers, discovering that only 1/3 monophages was able to translocate into the liver suggests that CJLB-5 and CJLB-14 are ineffective and therefore, reduce the overall efficacy of CampyShield. Considering this, as mentioned in the previous chapter, replacing CJLB-5 with CP30A may improve the efficacy of CampyShield in the treatment of campylobacters and the impact on the liver. Likewise, replacing CJLB-14 instead, with CP30A may also improve the efficacy of CampyShield to limit *Campylobacter* translocation to the livers of broiler chickens. To test this hypothesis, future studies would involve further testing of all cocktail phages and liver phages on *Campylobacter* strains that have demonstrated to translocate from the gut to the liver.

Chapter 5- *In vitro* investigations of CampyShield

5.1. Introduction

Campylobacter spp. poses a major threat to global healthcare, being the leading cause of bacterial gastroenteritis and one of the most identifiable bacteria to cause an infection that precedes the development of Guillain-Barré syndrome. Guillain-Barré cases caused by *C. jejuni* infection are usually the most severe compared to those by other causes (Finsterer, 2022). *Campylobacter* is also the cause of several other major health concerns such as Miller Fisher syndrome, inflammatory bowel disease, colorectal cancer and brain abscesses (Kaakoush *et al*., 2015). These detrimental effects to human health highlight the urgency for utilisation of suitable therapeutics in the farming process, to prevent and combat *Campylobacter*-related diseases. As mentioned previously, despite antibiotics being the most common treatment method against bacterial diseases, the ubiquitous colonisation of broiler chickens by *Campylobacter* makes such treatments impractical and likely more detrimental to human health as antibiotic resistance increases. Due to the consumption of undercooked poultry being the most common transmission route to humans, it is essential that treatment methods comprise of effective decolonisation strategies on poultry farms and highlights the importance of introducing effective bacteriophages to the market to control *Campylobacter* before it can be transmitted to humans.

The efficacy of bacteriophages for commercial use is determined by several factors. These include the extent of the phage host range, efficiency of plating, reduction in bacteria counts and their stability in the environment (Glonti and Pirnay, 2022; Mirzaei and Nilsson, 2015). This chapter outlines several *in vitro* investigations that were conducted in support of application studies with the CampyShield phage cocktail. Investigations included: discovering detection limits, host ranges, EOP values and multiplicity of infection (MOI) values. Phages were compared individually and as the cocktail, to identify what the most successful application method might look like. Resistant campylobacters were also identified after exposing them to CampyShield over 24 h; they were tested with the cocktail phages and DNA sequencing was used to determine possible resistance mechanisms.

5.2. Results

5.2.1. Detection limits of CampyShield phages and CP30A on *C. jejuni* PT14

CampyShield phages and CP30A were subjected to titrations to determine the limit of detection on *C. jejuni* PT14. Original stocks contained 10^8 PFU ml^{-1}. Table 18 below displays the detection limits as log_{10} PFU values, with standard deviations. No detection limit was obtained for CJLB-14 as it is a group II phage that does not replicate well on *C. jejuni* PT14.

Table 18. Detection limits of CampyShield phages and CP30A after plating onto *C. jejuni* PT14. Standard deviations were recorded to 2 s.f.

Phage type	Mean log_{10} PFU	± SD
CJLB-5	1.59	0.08
CJLB-10	1.60	0.04
CJLB-14	-	-
CampyShield	2.41	0.12
CP30A	1.54	0.09

5.2.2. Testing host ranges of CampyShield and liver phages

CampyShield and three phages recovered from the livers of group T5 chickens (*C. jejuni* colonised birds treated with CampyShield) were subjected to dilution series and tested with different *Campylobacter* strains to investigate their host ranges. *C. jejuni* PT14, *C. coli* NCTC 12668 and *C. jejuni* GIIC8 were used as host bacteria to make the lawns. EOP values were also calculated for each phage.

Table 19 below shows the phage titres produced by all phages against each *Campylobacter* strain. The data revealed that all phages except CJLB-14 produced high titres against *C. jejuni* PT14 and that CJLB-14 was the only one to produce a higher titre than 10 plaques on *C. coli* 12668. CJLB-10 and the liver phages all produced high titres on *C. jejuni* GIIC8, suggesting that the liver phages are likely CJLB-10. CJLB-5 and the CampyShield cocktail only produced high titres on *C.*

jejuni PT14, which was expected. Lastly, the EOP values calculated for two of three liver phages indicated they were as effective against *C. jejuni* GIIC8 as they were against *C. jejuni* PT14, which is promising.

Table 19. Results from host range testing of CampyShield and liver phages on several *Campylobacter* strains. Phage titres and EOP values were calculated to assess phage efficacy. EOP values used titres on *C. jejuni* GIIC8 against *C. jejuni* PT14 titres.

	Reference phage titre (PFU ml^{-1})				**Phage recovered from liver**		
***Campylobacter* strain**	CJLB-5 (gp III)	CJLB-10 (gp III)	CJLB-14 (gp II)	CampyShield cocktail	P35	P38	P40
C. jejuni PT14	9 $\log_{10}$	9 $\log_{10}$	≤ 10 P	7 $\log_{10}$	≥ 100 P	≥ 100 P	≥ 100 P
C. coli 12668	≤ 10 P	≤ 10 P	5 $\log_{10}$	≤ 10 P	≤ 10 P	≤ 10 P	≤ 10 P
C. jejuni GIIC8	≤ 10 P	6 $\log_{10}$	≤ 10 P	≤ 10 P	≥ 100 P	10 < x < 100	≥ 100 P
EOP	10^{-8}	10^{-3}	1	10^{-6}	1	0.5	1

P = plaques.

5.2.3. Attempts to isolate resistant *C. jejuni* NCTC 12662

5.2.3.1. *Campylobacter* counts

A *C. jejuni* NCTC 12662 laboratory strain that permits the replication of the CampyShield phages was infected with the cocktail monophages over 24 h in attempt to isolate cocktail-resistant colonies and to assess the ability of the CampyShield constituent phages to hold back the growth of the host bacteria. *Campylobacter* counts and phage titres in the control and test cultures were calculated over the culturing period. The colonies obtained at T = 24 h were then used to create lawns to test resistance against the CampyShield constituent phages. Figures (6.1-6.3) display the *Campylobacter* counts over the 24 h period for the monophages: CJLB-5, CJLB-10 and CJLB-14. Tables (20.1-20.3) display the mean counts of the monophage cultures, with standard deviations, from T = 0-24 h. They also indicate the initial MOI calculated and the T-test result from T = 6 h and T = 24 h for the monophages.

The raw data in figures 6.1. and 6.2. shows that CJLB-5 and CJLB-10 were effective in reducing *Campylobacter* counts after the 24 h period. The difference in results were also statistically significant. Figure 6.3. shows that CJLB-14 has quite a different effect on this *Campylobacter* strain compared to the other two monophages, with infected counts being lower than control counts and producing statistically significant results, until T = 24 h, when they are roughly the same and results are not significant. Furthermore, resistance was detected only by cultures infected with CJLB-10 and CJLB-14.

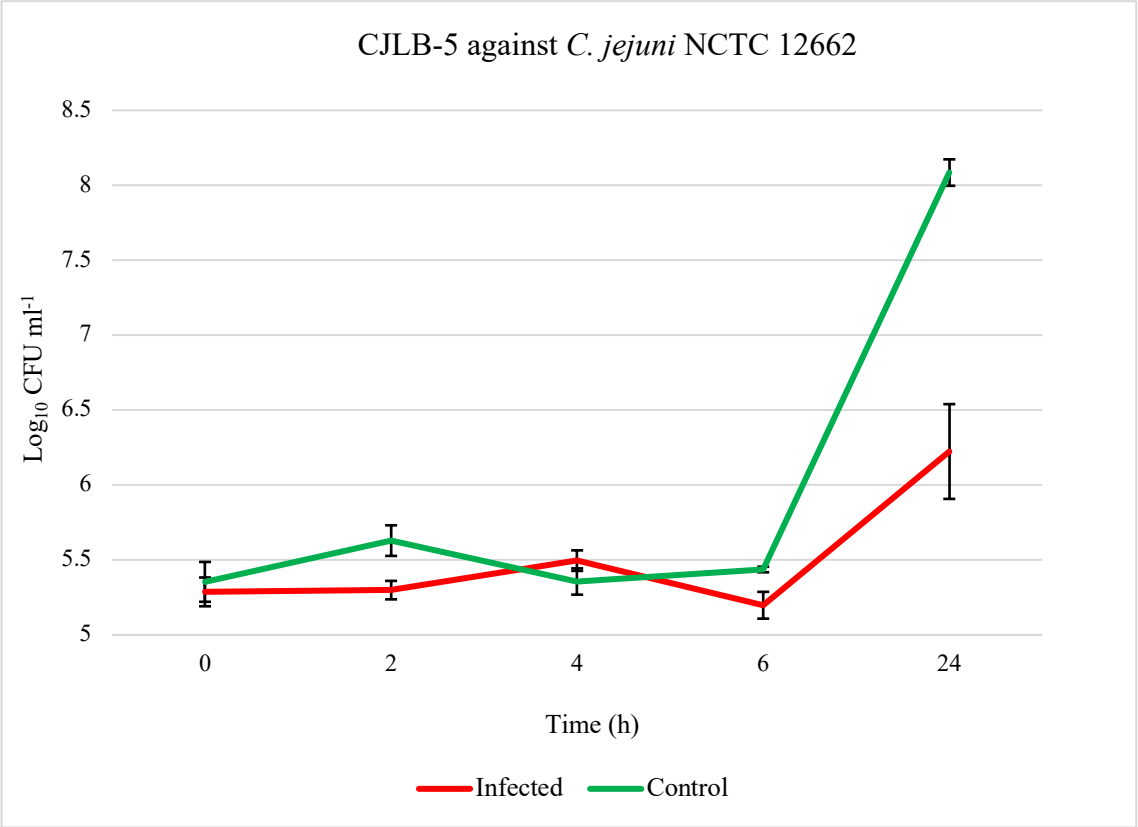

Figure 6.1. *Campylobacter* counts of *C. jejuni* NCTC 12662 when exposed to CJLB-5 over 24 h. Counts are recorded in $\log_{10}$ form and standard error bars are displayed.

Table 20.1. CJLB-5 mean $\log_{10}$ CFU ml^{-1} values for infected and control cultures, with standard deviations. The MOI value was calculated at T = 0 h. Significant differences between infected and control counts were calculated using ANOVA with Tukey's Honest Significant Difference (HSD) with significance set at 0.05.

Time (h)	Mean ± SD infected	Mean ± SD control	T-Test (P value)	MOI
0	5.29 ± 0.096	5.35 ± 0.13	-	
2	5.30 ± 0.06	5.63 ±0.10	-	
4	5.50 ± 0.07	5.34 ± 0.09	-	50.8
6	5.20 ± 0.09	5.44 ± 0.02	0.01061	
24	6.22 ± 0.32	8.09 ± 0.09	0.00018	

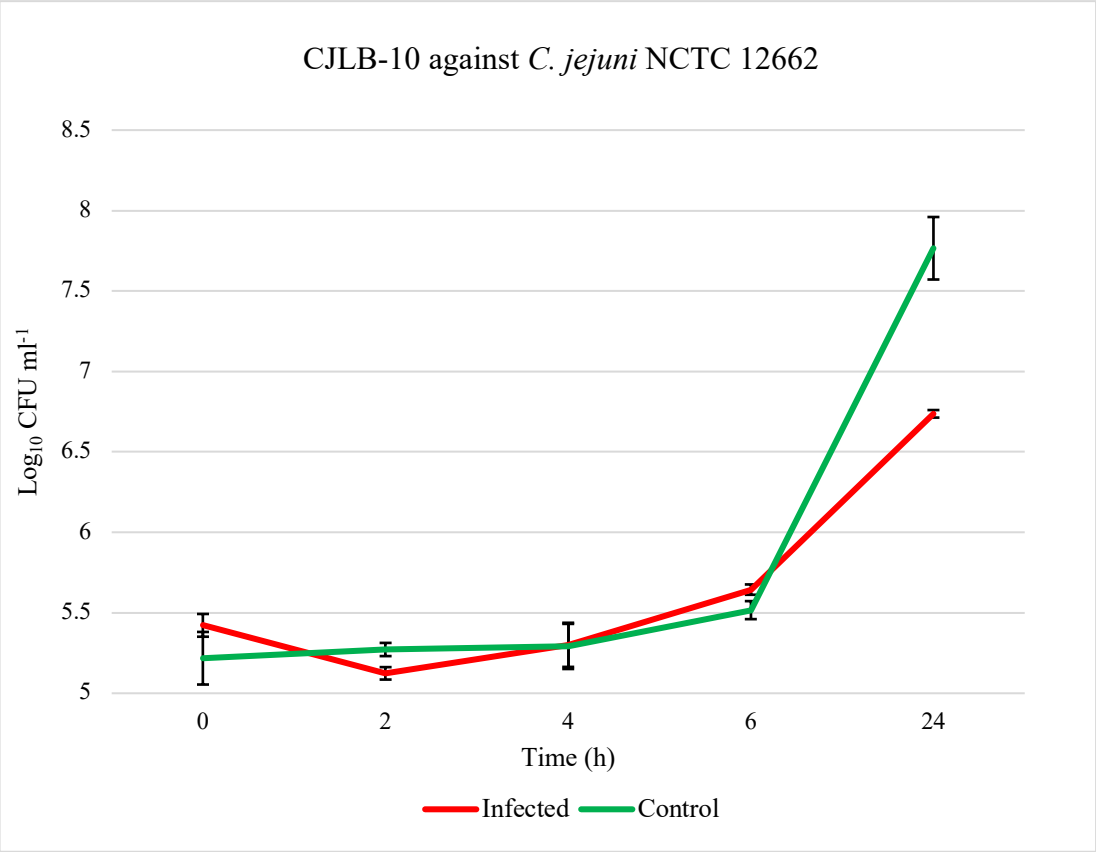

Figure 6.2. *Campylobacter* counts of *C. jejuni* NCTC 12662 when exposed to CJLB-10 over 24 h. Counts are recorded in $\log_{10}$ form and standard error bars are displayed.

Table 20.2. CJLB-10 mean $\log_{10}$ CFU ml^{-1} values for infected and control cultures, with standard deviations. The MOI value was calculated at T = 0 h. Significant differences between infected and control counts were calculated using ANOVA with Tukey's Honest Significant Difference (HSD) with significance set at 0.05.

Time (h)	Mean ± SD infected	Mean ± SD control	T-Test (P value)	MOI
0	5.42 ± 0.071	5.22 ± 0.16	-	
2	5.12 ± 0.039	5.27 ± 0.041	-	
4	5.301 ± 0.14	5.29 ± 0.14	-	
6	5.64 ± 0.032	5.52 ± 0.056	0.0123	37.5
24	6.74 ± 0.023	7.77 ± 0.19	0.0002	

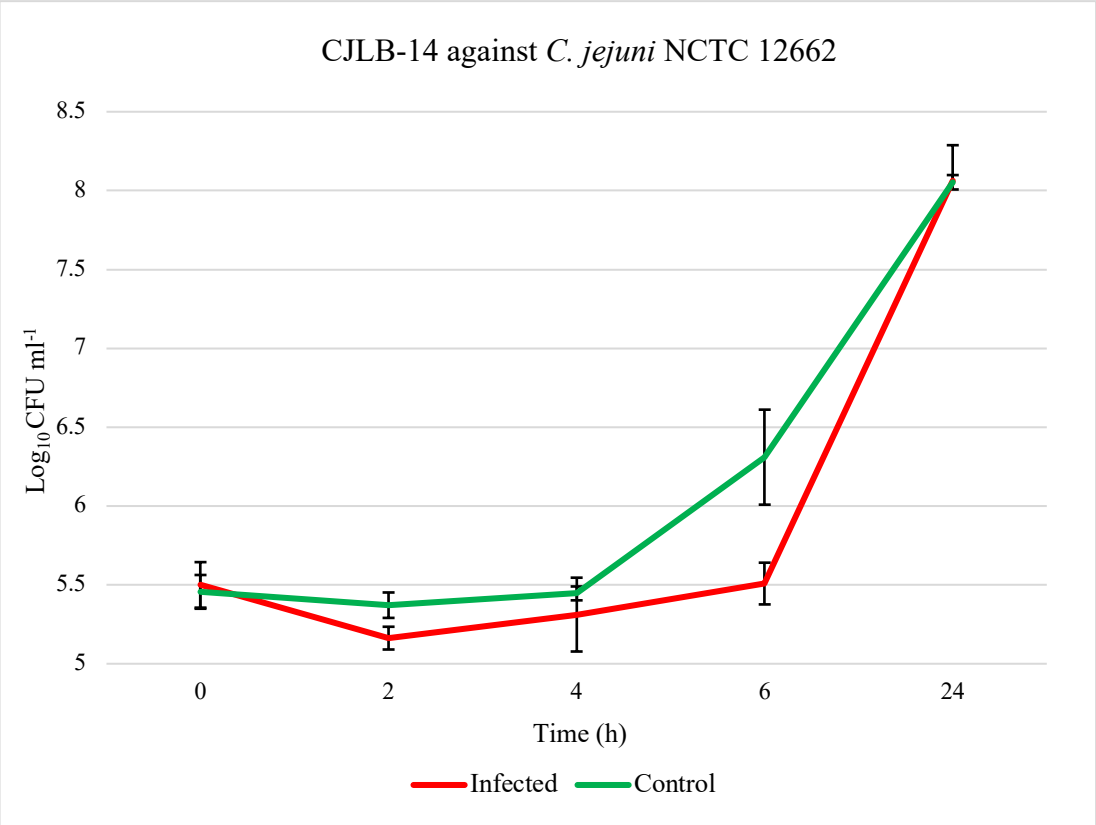

Figure 6.3. *Campylobacter* counts of *C. jejuni* NCTC 12662 when exposed to CJLB-14 over 24 h. Counts are recorded in $\log_{10}$ form and standard error bars are displayed.

Table 20.3. CJLB-14 mean $\log_{10}$ CFU ml^{-1} values for infected and control cultures, with standard deviations. The MOI value was calculated at T = 0 h. Significant differences between infected and control counts were calculated using ANOVA with Tukey's Honest Significant Difference (HSD) with significance set at 0.05.

Time (h)	Mean ± SD infected	Mean ± SD control	T-Test (P value)	MOI
0	5.50 ± 0.14	5.46 ± 0.11	-	30.8
2	5.16 ± 0.072	5.37 ± 0.081	0.0188	
4	5.31 ± 0.23	5.45 ± 0.044	-	
6	5.51 ± 0.13	6.31 ± 0.30	0.0039	
24	8.062 ± 0.23	8.053 ± 0.046	-	

5.2.3.2. Phage titres

Infected monophage samples were subjected to phage enumeration after completing the growth curve to identify any reductions or increases compared to what was initially added. Mean log_{10} PFU ml^{-1} counts with standard deviations are displayed. Two-tailed, two sample with equal variance T- tests were conducted to identify differences between original titres (Table 21) and post-experiment titres (Table 22).

Table 21. Titrations of cocktail phages onto *C. jejuni* NCTC 12662 before the growth curve experiment. Titres are recorded as mean log_{10} PFU ml^{-1} with standard deviations.

Phage	**Mean (log_{10} PFU ml^{-1}) ± SD**
CJLB-5	9.45 ± 0.12
CJLB-10	7.41 ± 0.030
CJLB-14	9.81 ± 0.072
CampyShield	7.85 ± 0.060

Table 22. Titrations of phage at each time point during infection of *C. jejuni* NCTC 12662 with P-values compared to the initial titre. Significant differences between infected and control counts were calculated using ANOVA with Tukey's Honest Significant Difference (HSD) with significance set at 0.05. NS = not significant.

Phage	Time (h)	Mean ± SD	T-Test (P value)
CJLB-5	0	6.52 ± 0.16	-
	2	6.64 ± 0.030	NS
	4	6.45 ± 0.084	NS
	6	6.52 ± 0.039	NS
	24	6.90 ± 0.027	0.014
CJLB-10	0	6.89 ± 0.043	-
	2	5.60 ± 0.089	< 0.001
	4	5.47 ± 0.073	< 0.001
	6	4.86 ± 0.046	< 0.001
	24	7.18 ± 0	< 0.001
CJLB-14	0	6.93 ± 0.026	-
	2	6.94 ± 0.063	NS
	4	6.87 ± 0.029	NS
	6	6.68 ± 0.026	< 0.001
	24	6.91 ± 0.015	NS

5.2.3.3. Resistant isolate lawns for testing against phages

Several colonies were isolated from infected T = 24 h plates and were sub-cultured to make lawns for resistance testing towards CampyShield and the monophages. Colonies were tested with the phage they had been exposed to in the experiment and the results were recorded as the EOP compared to the wild type *C. jejuni* NCTC 12662 (Table 21). Resistance was determined based on the absence of plaques/lysis as the EOPs were $< 10^{-5}$. Isolates were labelled I as they were from infected cultures and numbered 1-3 due to triplicate testing. Letters denote the colony number as five replicates were taken from each culture for enumeration.

Table 23 shows that at T = 24 h, all isolates were resistant to the phages they were tested against, except those against CJLB-5.

Table 23. Resistance testing results of T = 24 h colonies against their respective phage.

Phage	Isolate	EOP	Sensitive/resistant (S/R)
CJLB-5	I3A	1	S
CJLB-10	I2A	$< 10^{-5}$	R
	I3A	$< 10^{-5}$	R
	I1A	$< 10^{-5}$	R
CJLB-14	I1B	$< 10^{-5}$	R
	I2A	$< 10^{-5}$	R
	I1A	$< 10^{-5}$	R
CampyShield	I2A	$< 10^{-5}$	R
	I3A	$< 10^{-5}$	R
	I3B	$< 10^{-5}$	R

5.2.3.4. DNA sequencing

Four of the nine resistant isolates shown above were chosen for DNA isolation and sequencing. The Illumina MiSeq system was used for sequencing and the reads obtained were mapped to the genome of wild type *C. jejuni* NCTC 12662. Table 24 outlines which mutations were observed in each of the sequenced isolates. Table 25 provides the details of the mutations found in the resistant isolates.

There were many mutations identified in the resistance isolates, shown in Table 25, and all the isolates possessed several mutations, as seen in Table 24. These include changes in metabolism and DNA synthesis and changes in motility such as the bacteria becoming non-motile, or differences in the flagella. Mutations also lead to changes in invasion proteins (potentially to evade invasion), changes in the assembly and structure of surface structures, changes in the lipopolysaccharide, CPS, chemotaxis, nutrient uptake and in communication, leading to enhanced survival. These mutations could have functions associated with phage recognition and completion of the infection cycle.

Table 24. Sequenced *C. jejuni* NCTC 12662 resistant isolates and the mutations associated with them, with reference to column 1 of Table 25 'Mutation reference no.'.

***C. jejuni* NCTC 12662 resistant isolate**	**Associated mutations**
CJLB-10/ I3A	2, 3, 8, 12, 13, 15, 16, 17, 18
CJLB-14/ I1A	1, 3, 4, 5, 8, 11, 12, 13, 15, 16, 17, 18
CJLB-14/ I2B	3, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18
CampyShield/ I2A	3, 5, 8, 12, 13, 15, 16, 17, 18

Table 25. Observed nucleotide changes in the genome sequences specific to the *C. jejuni* NCTC 12662 resistant isolates, compared to database wild type *C. jejuni* NCTC 12662.

Mutation reference no.	Reference position	Accession number	Frequency	Mutation type	Amino acid change	Gene product	Resistance explanation
1	74656	B2K12_00265	75.4	Insertion	B2K12_00265:p.Asp239fs	polyG 9G variant at C-term normally 8G, annotation suggests a pseudogene but iron-binding protein reading frame largely intact	Iron-binding protein no longer coded for. Can lead to changes in metabolism and DNA synthesis and changes in surface structures
2	107865	B2K12_00440	94.5	Deletion	AQX68602.1:p.Lys124fs	C4-dicarboxylate ABC transporter can be pseudogene in some strains	Membrane-bound protein required for metabolism. Phage target protein no longer coded for

3	156719	B2K12_00725	99.7	Insertion	B2K12_00725:p.Lys95fs	Annotated as chemotaxis pseudogene	Chemotaxis protein no longer coded for. Can lead to changes in motility
4	313931	B2K12_01615	77.1	Deletion	AQX68825.1:p.Ile141fs	polyA 7A variant normally 6A, flagellar biosynthesis protein FlhB - the mutation is non-motile	Isolate is non-motile so evades infection by phages that target the flagella
5	642503	B2K12_03325	97.4	Deletion	B2K12_03325:p.Pro282fs	polyC 9C variant normally 10C, this appears to put it in frame i.e. functional annotated as invasion protein but not characterized, DUF2972 domain	Uncharacterised protein but perhaps mutation causes a change in an invasion protein
6	814492	B2K12_04195	96.4	SNV	AQX69289.1:p.Gln640	Stop codon in flagellar biosynthesis	Isolate is non-motile so evades infection by phages

						protein FlhA, not motile	that target the flagella
7	990230	B2K12_05085	97.5	SNV	AQX69449.1:p.Glu241Lys	Point mutation in aspartyl/glutamyl-tRNA amidotransferase subunit A	Changes in production of correctly charged Gln-tRNA (Gln) may lead to misfolded or nonfunctional phage receptors
8	1217971	B2K12_06235	88.4	Insertion	AQX69666.1:p.Ile52fs	polyG 8G phase off normally 7G aminopeptidase	Changes in assembly of surface structures recognised by phages
9	1235201	B2K12_06335	77.6	Insertion	AQX69684.1:p.Tyr60fs	polyG 10G phase off normally 9G PseD/Motility Accessory Factor for post-translational	Changes in motility, lead to evasion of flagella-targeting phages

						glycosylation of flagellar proteins	
10	1239662	B2K12_06350	80.6	Insertion	B2K12_06350:p.Asn99fs	polyG 11G variant normally 10G, annotated as formyl transferase pseudogene	Formyltransferase mutation has been seen to cause changes in the lipopolysaccharide
11	1281402	N/A	81.3	Deletion	N/A	polyC 8C variant normally 9C but non-coding appears to be disrupted C4 dicarboxylic acid transporter	Protein required for metabolism. Phages sometimes rely on metabolic pathways. Potential changes in surface structures
12	1288636	B2K12_06565	87.3	Insertion	AQX69723.1:p.Glu513_Leu514insAsnLeuGlu	Nucleotidyltransferase reading frame intact with 3 extra amino acids from 9 bases, which are similar to other database entries	Altered lipo-oligosaccharides causes changes in CPS

13	1335297	B2K12_06795	89.1	Insertion	AQX69768.1:p.Asn561fs	Disrupted PEP-utilizing enzyme	PEP is important for glycolysis so may lead to changes in metabolism
14	1348832	B2K12_06855	87.0	Deletion	AQX69779.1:p.Tyr531fs	polyG 8G variant normally 9G, sugar transferase	Changes in sugar transferases lead to altered CPS
15	1377186	B2K12_07000	97.1	Insertion	AQX69806.1:p.Val17fs	polyT 8T variant normally 7T hypothetical protein but this restores TSUP family transporter reading frame	Transport systems are involved in nutrient uptake and communication, resulting in enhanced survival
16	1399947	B2K12_07110	99.0	Deletion	AQX69828.1:p.Lys167fs	Loss of T frameshifts CheY-P-specific phosphatase CheX	CheX can cause a reduction in effect of CheY on flagellar rotation or can cause changes in chemotaxis

17	1400304	B2K12_07110	92.7	Insertion	AQX69828.1:p.Lys46_Lys48dup	Insertion upstream of frameshift in CheY-P-specific phosphatase CheX of with 3 extra amino acids from 9 bases	CheX can cause a reduction in effect of CheY on flagellar rotation or can cause changes in chemotaxis
18	1575872	B2K12_08060	100	SNV	AQX70002.1:p.Met276Ile	Point mutation in type II citrate synthase	Involved in TCA cycle. Changes may result in altered metabolism

PEP = phosphoenolpyruvate. TCA= Tricarboxylic acid

5.3. Discussion

Investigations into the detection limits of the cocktail phages and CP30A against the universal strain *C. jejuni* PT14 allowed initial conclusions to be drawn regarding the stability of CampyShield and the component monophages. Phage CJLB-14 produced expected results as a group II phage, with activity against *C. coli* NCTC 12668 but low or no activity against *C. jejuni* PT14. This will be a contributory factor to the CampyShield detection limit being recorded as 0.8 $\log_{10}$ PFU greater than that of CJLB-5, CJLB-10 and CP30A. Phages CJLB-5, CJLB-10 and CP30A produced similar detection limits, suggesting this combination may produce a similarly active and stable cocktail. The comparable detection limit of CJLB-14 against *C. coli* NCTC 12668 was 2.9 $\log_{10}$ PFU based on the specification of the manufacturer. Although a cocktail containing CJLB-5, CJLB-10 and CP30A may prove more stable, it is necessary that a phage cocktail comprises of monophages belonging to group II and group III, to extend its host range. These two groups are different in their strain targeting and host cell receptors therefore, joint activity of group II and group III phages would be optimal for targeting *C. jejuni* and *C. coli* and might enhance treatment efficacy and reduce the number of resistant bacteria (Steffan *et al*., 2022 and Hammerl *et al*., 2014). However, it is difficult to draw final conclusions as *in vitro* investigations do not always replicate *in vivo* results.

Host range testing was carried out for CampyShield and liver phages isolated from enrichment testing. As seen in Table 19, all phages, except CJLB-14, produced high titres on *C. jejuni* PT14. Only CJLB-14 produced a high titre on *C. coli* NCTC 12668, indicating that the host ranges of the other phages may not extend to *C. coli*, which reduces their efficacy, particularly in the cocktail. CJLB-10 and the liver phages all infected *C. jejuni* GIIC8 to a high level, indicating their host range extended well to this strain. However, the efficiency of plating value for CJLB-10 at 10^{-3} demonstrated that infection of *C. jejuni* GIIC8 by this phage was less successful compared to infection of *C. jejuni* PT14. The liver phages produced much higher EOPs on *C. jejuni* GIIC8, at 1, 0.5 and 1, highlighting their efficacy towards this strain. On the other hand, the low EOP values observed by CJLB-5 and CampyShield towards *C. jejuni* GIIC8 indicate their host ranges fail to extend to this strain. All the cocktail phages also successfully infected *C. jejuni* NCTC 12662, as

shown in Table 21. Based on these results, CJLB-10 is the only phage out of the cocktail phages to infect more than two strains, indicating the others seem to have narrow host ranges. This is not promising for the therapeutic use of CampyShield as the inclusion of therapeutic phages in a cocktail should have a broader host range, aiming for more than 30% of isolates El-Shibiny *et al*., (2009). Phages such as CP30A and CP6 have been recorded to have broad host ranges. Zhang *et al*., (2024) reported that CP6 exerted good antimicrobial effects on multi-drug-resistant campylobacters and reduced colony counts by up to 1-log. In addition, CP6 showed lytic activity against 38/39 *Campylobacter* isolates, including both *C. jejuni* and *C. coli*. Results obtained from the liver phages, in Table 19, also indicated that CJLB-10 was the only phage that translocated into the liver from the caecum as the results aligned with successful plaque formation on *C. jejuni* GIIC8. Further studies would be necessary to test the phages on more *Campylobacter* strains as they were only tested on three here. This does not allow for final conclusions to be drawn regarding the full extent of host ranges.

Resistant strains of *C. jejuni* NCTC 12662 were isolated after 24 h of infection with CampyShield monophages. Resistance within bacterial populations may occur as they rapidly mutate to survive in the harsh conditions they have been exposed to for a long period of time, resulting in changes to fitness and virulence. They can evolve mechanisms such as changing their structure, to evade bacteriophage infection (Hasan and Ahn, 2022). Cultures infected by CJLB-5 were the only ones to produce sensitive strains as seen in Table 23. This is supported by the data shown in Figure 6.1., where *Campylobacter* counts are lower in infected samples at T = 6 h but much lower in infected samples at T = 24 h, compared to control samples, by ~ 2 $\log_{10}$ CFU ml^{-1}. P values at T = 6 h and T = 24 h, of 0.01061 and 0.00018 respectively, indicate that these counts are significantly different to those obtained from control cultures ($P < 0.05$). However, this does not mean there were no resistant strains present as only a limited number of strains were tested and the *Campylobacter* count still increases in the infected cultures by ~1 $\log_{10}$ CFU ml^{-1} from T = 6 h to T = 24 h.

Cultures infected by CJLB-10 display lower infected *Campylobacter* counts after T = 24 h, compared to control cultures, by ~ 1 $\log_{10}$ CFU ml^{-1}, as shown in Figure 6.2., which was lower than that by CJLB-5. Higher counts with infected cultures at T = 6

h are significant (P = 0.0123), which is not promising in terms of phage efficacy. However, the lower counts at T = 24 h are more significant (P = 0.0002). Resistant strains were isolated from these cultures, which is consistent with the results from Figure 6.2., as the infected counts are higher than the control counts at T = 6 h. Perhaps a 1 log $_{10}$ difference in count reductions seen between CJLB-10 and CJLB-5 could be influenced by the lower MOI value of 37.5 for CJLB-10 compared to 50.8 for CJLB-5. A higher ratio of CJLB-5 phage particles to *Campylobacter* cells were added, meaning more of the campylobacters could be infected by phage, resulting in lower counts. On the other hand, CJLB-5 seems to be a more effective phage at infecting *C. jejuni* NCTC 12662 as results from Table 21 reveal a plaque titre of 2 $\log_{10}$ higher compared to that of CJLB-10.

The effect of CJLB-14 on *C. jejuni* NCTC 12662 seems to be greater over the first 6 hours, compared to CJLB-5 and CJLB-10. The lower infected counts are significantly different from the control counts at T = 2 h and T = 6 h; P = 0.0188 and P = 0.0039, respectively. However, once 24 h is reached, the counts are not significantly different. Infected *Campylobacter* counts are around the same as control counts, at just above 8 $\log_{10}$ CFU ml^{-1}. This suggested that a high proportion of the population became resistant to CJLB-14 and DNA sequencing was used to investigate the potential reasons for this. In addition, likewise with CJLB-10, the MOI was lower than 37.5 and much lower than 50.8, which could influence the number of colonies infected by CJLB-14, as this phage produced a higher titre than CJLB-5 against *C. jejuni* NCTC 12662, as shown in Table 21.

The fact that limited numbers of resistant *C. jejuni* NCTC 12662 strains were isolated from CJLB-5 cultures is promising as the development of phage resistance limits the use of phages when controlling *Campylobacter* counts (Al Hakeem *et al*., 2022). Furthermore, this shows that CJLB-10 and CJLB-14, especially, may not be so effective in phage therapy due to the higher numbers of resistant campylobacters isolated post infection. However, final conclusions cannot be made surrounding this hypothesis as only one strain was used in this experiment and different MOIs were applied. In further studies, an effort should be made to obtain the same MOI for all tests to make more accurate comparisons. In addition, CJLB-14 is a group II phage, which has more limited infectivity against *C. jejuni* strains. Furthermore, as

mentioned previously, these investigations were carried out in vitro which provides different conditions to those experienced *in vivo*.

Phage titres against wild type *C. jejuni* NCTC 12662 were calculated to allow for comparison with phage titres calculated after resistant colonies were isolated. The phage titre of CJLB-5 at T = 24 h yielded a significant difference ($P = 0.014$), likewise with titres of CJLB-10 at T = 2-24 h ($P < 0.001$) and CJLB-14 at T = 6 h ($P < 0.001$). These values indicate significant reductions in the phage titres after isolating resistant *C. jejuni* NCTC 12662 colonies. These significant reductions indicate that the concentrations of these phages could still interact with the host bacteria after 24 h, experiencing reductions of up to 2.55 $\log_{10}$ CFU ml^{-1}.

The results in Table 23 show the efficiency of plating values of the monophages after *C. jejuni* NCTC 12662 resistant isolates were obtained. CJLB-5 was the only monophage that could be classed as producing a high production efficiency (Mirzaei and Nilsson, 2015), with an EOP value of 1. However, this is only based on testing against one isolate so further testing with multiple isolates would provide more reliable results regarding the efficiency of plating of phage CJLB-5. Phages CJLB-10 and CJLB-14 would be considered inefficient according to Mirzaei and Nilsson (2015), as they produced EOP values of $< 10^{-5}$. In addition, all the strains tested against them were resistant therefore, the extent of resistance was too high. These results present issues regarding the overall efficacy of the CampyShield constituent phages (except phage CJLB-5 in this sense) as inefficient EOP results are not acceptable in therapeutic applications, likewise with high mutation frequencies.

DNA sequencing was carried out for the four resistant isolates shown in Table 24, to identify phase variation, experienced *in vitro*, that was likely responsible for resistance towards the CampyShield monophages. Mutation 1 resulted in changes to an iron-binding protein, leading to potential alterations in the downstream gene that may be involved in motility (Cayrou *et al*., 2021). In addition, changes in iron levels may influence the expression of cell surface receptors, impacting phage binding.

Mutations 2 and 11 involved changes in a C4-dicarboxylate transporter, which is a membrane-bound protein required for metabolism. They are involved in offering the

carbon and energy source in bacterial metabolism and growth (Janausch *et al*., 2002). This might have led to phage resistance due to changes in the uptake of metabolites; if uptake is enhanced, the fitness of *C. jejuni* under stressful conditions is improved, along with their ability to resist phage infection. Mutation 13, a disruption in a PEP-utilising enzyme, may have also led to changes in metabolism as this protein is important in glycolysis (Hofreuter, 2014). Furthermore, mutation 18 was a point mutation in a type II citrate synthase which is involved in the TCA cycle and metabolism (Weingarten *et al*., 2009).

Mutations 3, 16 and 17 involved phase variation of chemotaxis-associated genes. Chemotaxis directs flagellar motility in *C. jejuni* in response to environmental stimuli. CheY transmits the signal to the flagellar switch, leading to changes in flagellar rotation and CheX can cause a reduction in the effect of CheY when altering flagellar rotation and motility (Reuter *et al*., 2020), as it regulates chemotaxis by dephosphorylating CheY-P. Therefore, the frameshifts in CheX caused by mutations 16 and 17 led to resistance among *C. jejuni* through the loss of motility as CheY was unable to transmit signals to the flagellar switch. Mutations in motility are strongly linked to resistance due to the inability of some phages to bind to the flagella (Gencay *et al*., 2018). Gencay *et al*., (2018) stated that earlier studies indicated this resistance through loss of motility after using a transposon library of *C. jejuni* NCTC11168. In addition, changes in these proteins can promote the formation of biofilms by *C. jejuni*, which support survival (Reuter *et al*., 2020) through evading phage infection.

Likewise, mutations 4, 6 and 9 lead to further changes in motility. Mutation 4 in the FlhB protein caused the isolate to be non-motile as there was a frameshift causing a change from poly 6A to 7A. Although FlhB does not impair motility as much as the FlhA protein, a study by Inoue *et al*., (2019) has also observed significantly reduced motility by a mutation in an *flhB* gene. Mutation 6 resulted in a stop codon in the FlaA flagellar biosynthesis protein which caused non-motility. FlhA is the most important part of the flagellar machinery as it is the key component in the flagellar export apparatus and a pivotal stage in flagellar biosynthesis is the ability to export structural components (Carrillo *et al*., 2004). Therefore, mutations in *flhA* results in the isolate being non-motile, as seen by *C. jejuni* NCTC11168 (Gencay *et al*., 2018)

and subsequently able to evade infection by certain phages. Mutation 9 led to changes in post-translational glycosylation of flagellar proteins and as mentioned in chapter 3, glycosylation is involved in structure modification. Therefore, modifying the flagellar proteins located on the bacterial surface alters the ability of phage to bind to the surface receptors. As mentioned, changes in motility are usually associated with resistance towards group II phages (Sørensen *et al*., 2015). However, Lis and Connerton (2016), identified that changes in motility can also confer resistance towards group III phages. All *C. jejuni* NCTC 12662 resistant isolates experienced mutations that caused changes in motility and these isolates were resistant to both CJLB-10 and CJLB-14 phages which are group III and group II, respectively.

The effects of mutation 5 are unknown as the DUF2972 protein is uncharacterised; investigations into this protein would require further studies so that appropriate conclusions can be drawn. However, this mutation may cause changes in an invasion protein, leading to changes in CPS structures, conferring phage resistance.

Mutation 7 was a point mutation in an aspartyl/glutamyl tRNA amidotransferase, which are essential for accurate protein synthesis (Raczniak *et al*., 2001). This may have led to changes in the production of correctly charged Gln-tRNA which has the potential to lead to misfolded or nonfunctional phage receptors. Mutation 8 lead the turning of an aminopeptidase gene from phase ON to phase OFF. This may have led to changes in the assembly of surface structures as aminopeptidases are involved in the catabolism of external peptides and are vital in the final steps of protein turnover (Gonzales and Robert-Baudouy, 1996).

Mutation 10 resulted in phase variation of a formyltransferase protein, which has been observed to cause changes in lipopolysaccharides on the bacterial cell surface. Li *et al*., (2019), recorded that inactivation of a formyltransferase gene was a significant factor in producing a rough phenotype in some bacterial species. Therefore, changes in the outer structure of *C. jejuni* would enable phage evasion due to the inability of phage to recognise common surface receptors. Mutations 12 and 14 also caused changes in the CPS through altered lipopolysaccharides and changes in sugar transferases.

Mutation 15 led to a frameshift mutation, changing a polyT 7T to 8T, which is involved in restoring a Toluene Sulfonate Uptake Permease **(**TSUP) family transporter reading frame. The TSUP family is thought to catalyse the transport of sulphur-based compounds and is therefore likely to be involved in nutrient uptake. These transport systems also play a role in metabolite excretion and intracellular communication through transporting signalling molecules (Shlykov *et al*., 2012). This can lead to phage resistance through potential enhanced nutrient uptake, enabling survival in harsh conditions.

The efficacy of the CampyShield cocktail based on these *in vitro* investigations is quite low. The host ranges of the CampyShield constituent phages were quite narrow, with only CJLB-10 successfully infecting more than just one strain and broad host ranges are vital for successful therapeutic phages. However, the fact that CJLB-14 infected *C. coli* 12668 is quite promising as it shows the constituent phages in CampyShield can infect different species. Yet, the infection of CampyShield on *C. coli* 12668 was hindered due to the inability of CJLB-5 and CJLB-10 to infect this strain. It is necessary to include a group II phage in the cocktail to extend host ranges so perhaps a more stable group III phage towards *C. jejuni* PT14 would compensate for the poor replication of a group II phage on this strain. The further testing of CampyShield and its constituent phages on more host strains was beyond the scope of this study but would be necessary in future research to fully grasp the extent of its host range, and therefore to make final conclusions on its efficacy. The EOP of the liver phages towards GIIC8 was much better than CJLB-10 *in vitro*. The hypothesis that these liver phages are CJLB-10, due to the results in Table 19, may suggest that CJLB-10 is more successful in reducing *Campylobacter* counts in chicken livers, which is promising for phage therapy in broiler chicken livers. However, further testing would be necessary to calculate EOP values of the phages on different host strains to gauge the full effect of the cocktail on multiple *Campylobacter* strains.

Based on the results from *Campylobacter* counts of *C. jejuni* NCTC 12662 against the monophages and the results from Table 22, it is clear that CJLB-5 was the most effective phage towards resistant isolates of this strain. The acquisition of a high level of resistance from a ‘universal strain’ towards CJLB-10 and CJLB-14 phages is not a favourable result in terms of the efficacy of the cocktail as the reduction of

Campylobacter counts in a therapeutic setting is limited. In addition, DNA sequencing revealed many potential resistance mechanisms through phase variation, such as changes in chemotaxis, reduced/ non-motility and changes in the CPS. Therefore, further studies would need to be conducted to test the CampyShield cocktail, as a whole, against *C. jejuni* NCTC 12662 as it is more difficult for campylobacters to develop resistance to three phages simultaneously than it is to develop resistance to a single phage.

Chapter 6- Conclusion and further studies

Campylobacter spp. is a prevalent foodborne pathogen, particularly among poultry products, and is the most common cause of human gastroenteritis in the world (WHO, 2020). Its high level of resistance to human antibiotics underlines the urgent need of treatment methods for broiler chickens before it reaches consumers. The use of bacteriophages in reducing *Campylobacter* counts in broiler chickens has been studied extensively and they have been found to be very effective. However, the success of phage therapy is dependent upon multiple factors. Firstly, the use of bacteriophage cocktails is a much more suitable approach to using a single phage due to the rapid emergence of resistance in bacteria after they are exposed to phages (Fang *et al*., 2024). The selection of phages that comprise the cocktail is the most important factor that affects efficacy; the phages must have broad host ranges, high infectivity rates and experience low mutation frequencies. Additionally, successful phage cocktails would include both groups of phages, groups II and III to achieve maximum strain coverage (El-Shibiny *et al*., 2009).

Bacteriophage cocktails have been recorded, in the majority of cases, to be more successful than single phages in reducing *Campylobacter* counts. However, significant results from the broiler chicken trial showed that CP30A was more effective in reducing *Campylobacter* counts compared to CampyShield, by almost 1.5 $\log_{10}$ $CFUg^{-1}$. In addition, CP30A experienced a much lower mutation frequency of 24 %, among the campylobacters, compared to CampyShield, at 65 %. This initially indicated that the cocktail was not as effective as it should be for therapeutic application. Furthermore, Table 11 indicated a large number of mutation events, that conferred resistance mechanisms, experienced by the isolates after residing in the liver with CampyShield. As mentioned, this frequent development of resistance towards the cocktail greatly limits its use in controlling *Campylobacter* counts in broilers. This can also be said with the high-level resistance and high number of mutation events experienced by *C. jejuni* NCTC 12662 isolates towards the constituent phages in the cocktail (Table 25). Phase variation in bacterial motility and surface structures are the most common resistance mechanisms developed by bacteria towards phages (Sørensen *et al*., 2021). Therefore, it is not surprising that these mutations were observed. However, in terms of therapeutic application, the

mutation frequency experienced by CampyShield must be lower to experience higher success rates in reducing *Campylobacter* counts.

The efficacy of the cocktail in chicken livers appeared to be limited as results showed that only one of the constituent phages (CJLB-10) was able to translocate to the liver from the caeca. CP30A was also observed to translocate to the liver, showing that this phage is more effective in the liver than CJLB-5 or CJLB-14. Therefore, for future therapeutic applications in reducing *Campylobacter* counts in chicken livers, CJLB-10 and/or CP30A would be more favourable than CJLB-5 or CJLB-14. In addition, based on the EOP values obtained from the liver phages in Table 19, CJLB-10 seems effective in the liver against *C. jejuni* GIIC8. Therefore, CP30A alongside CJLB-10 would be effective in reducing these counts in chicken livers.

The host ranges of the CampyShield phages were quite narrow, which is not favourable for therapeutic phages as it means they are limited in the strains and species they can infect. A promising aspect was discovering that CJLB-14 extended infection to *C. coli* NCTC 12668. However, CampyShield phages were not tested against many strains so final conclusions surrounding the overall host range efficacy of CampyShield and its constituent phages cannot be drawn.

Further studies and adaptations would be necessary in several aspects of this research to cement final conclusions surrounding the efficacy of CampyShield in broiler chickens. Firstly, it would be useful to ensure subcultures and lawns are incubated under the same method throughout, to avoid variations in results due to potential differences in the gaseous environments. Secondly, the cocktail and its constituent phages must be tested on a wider range of bacterial strains, including those from different species, to fully grasp the extent of phage infection and determine a more accurate host range. Efficiency of plating values must then be determined to evaluate how successful the phages are at infecting these different strains and reducing *Campylobacter* counts. Furthermore, future studies should involve testing the CampyShield cocktail over 24 h against *C. jejuni* NCTC 12662 and investigating the level of resistance towards the whole cocktail, comparing mutation frequencies with its constituent phages. Phage burst size is also an important aspect in determining the

efficacy of phages; a high burst size increases the probability that phages reach the target bacteria and therefore, contributes to productive infection. A high burst size may also result in a lower risk of phage-resistant bacteria emerging if they can eliminate bacteria faster than they can replicate (Mirzaei and Nilsson, 2015). Therefore, future studies of this research would involve investigating the burst size of CampyShield to further conclude its efficacy.

Based on the limited efficacy of CampyShield observed in this study, future research would be necessary to investigate component phages that more successfully target the isolates used, to cause a greater reduction in *Campylobacter* infections and subsequently a bigger improvement in consumer health. Potential phages must produce a lower mutation frequency post-treatment, be responsible for larger reductions in *Campylobacter* counts in broiler chickens, have a broader host range and a high burst size to be considered successful therapeutic phages for *Campylobacter* treatment in broiler chickens.

Chapter 7- References

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