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Improving poultry meat safety and sustainability

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Campylobacter in poultry meat

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1 Introduction

Over the past half-century, global poultry meat production has grown dramatically, growing over 12-fold from 1961 to 2014 [67, 68]. Driven by the perception of a healthier, lower-fat nutritional profile, and less association with cardiovascular disease and cancer compared to red meat, poultry has become a preferred choice for more health-conscious consumers [49]. In the US, e.g., chicken consumption has significantly outpaced that of other meats, including beef and pork, achieving growth of 160% per capita between 1970 and 2022, while

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beef and pork have seen a 31% and 6% reduction, respectively [50, 51]. Major producers include the US (annual output of over 20 million tons), the European Union (EU) (around 19 million tons) China (18 million tons) and Brazil (13 million tons) [67]. Growth projections are from 82 million tons in 2005 to a potential 181 million tons by 2050, an average annual expansion of 1.82%. [68]. This growth has led to more intensive forms of poultry production and processing, whether in large, indoor barns containing thousands of birds or in large-scale, highly automated processing facilities [48]. However, these increases in the level and scale of production have also increased the potential risk of foodborne disease.

Foodborne diseases from pathogens such as *Campylobacter* can be traced back to a wide range of food and non-food sources [57]. However, data analysing foodborne illness in 2019 in the US implicate chicken in nearly two-thirds (64.7%) of non-dairy *Campylobacter* illnesses, and that 56.5% of human *Campylobacter* infections are attributable to contaminated poultry products, highlighting chicken as a significant source of campylobacteriosis [50, 54, 58, 59]. The name 'Campylobacter' is derived from ancient Greek, where 'kampylos' indicates 'curved' and 'baktron' indicates 'rod'. The *Campylobacter* genus is comprised of approximately 57 species, including a number of clinical and veterinary importance in causing disease (e.g. *Campylobacter jejuni* and *Campylobacter coli*). The genus is characterized by Gram-negative bacilli with distinctive spiral shapes and polar flagella enabling a corkscrew-like motion (Fig. 1) [1, 2].

The potential role of *Campylobacter* spp. as a human health risk can be traced back to the US in 1938 when the bacteria were first isolated during

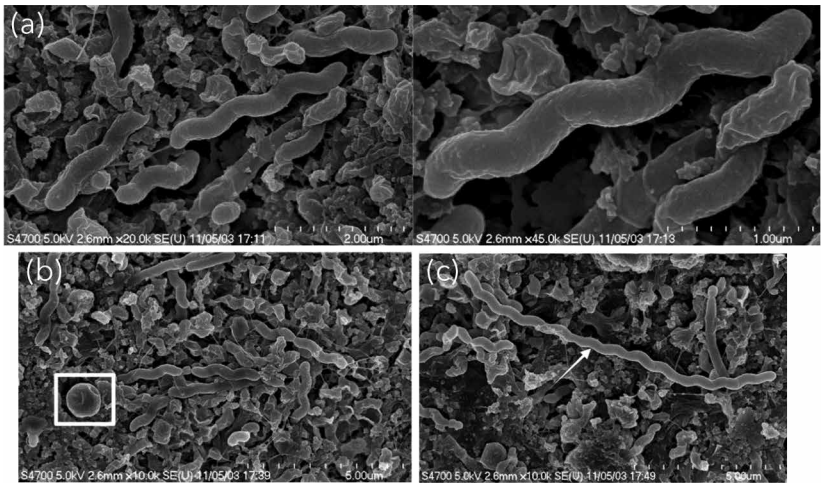

Figure 1 Morphology of *C. jejuni*. (a) helical-shaped *Campylobacter*; (b) viable but non-culturable form (coccoid) isolate is in the white frame box; (c) distinctive morphological deviation of a *Campylobacter jejuni* isolates (arrow).

a disease outbreak related to milk [3]. These bacteria were identified in the 1950s in the blood samples of children suffering from diarrhoea [3, 4]. A critical turning point in understanding a causal relationship between *Campylobacter* spp. and disease in humans came in Belgium in 1972 when they were isolated from the blood and faeces of a young woman who was previously healthy but had suddenly developed acute febrile hemorrhagic enteritis [5]. In 1977, Skirrow isolated the same organism from an infant with diarrhoea, prompting further investigation into the pathogenic potential of these bacteria which has continued ever since [6, 7]. Key developments in identifying and understanding the significance of *Campylobacter* as a pathogen are shown in Fig. 2.

The World Health Organization (WHO), together with Foodborne Disease Burden Epidemiology Reference Group (FERG), reported in 2020 that, out of an estimated 550 million foodborne illnesses per year, *Campylobacter* is identified as one of the top four global contributors to diarrhoeal diseases [8]. Their data indicates that *Campylobacter* was responsible for 166 million annual cases of diarrhoea and 31 700 cases of Guillain-Barré Syndrome (a neurological disorder), a problem exacerbated by the widespread distribution of multidrug-resistant strains of the pathogen [9–11, 26]. The latter issue highlights the complex interplay between environmental, animal, and human health which requires an integrated 'One Health' approach to disease surveillance, control, prevention, and mitigation [20, 33].

Campylobacteriosis due to contamination from poultry and other sources thus represents a significant public health challenge, frequently topping the list of reported foodborne illnesses. Health authorities in Europe and the US report around 230 000 and over 1.5 million annual cases, respectively, with an economic burden estimated in the US at around $6.9 billion [52, 53]. Other

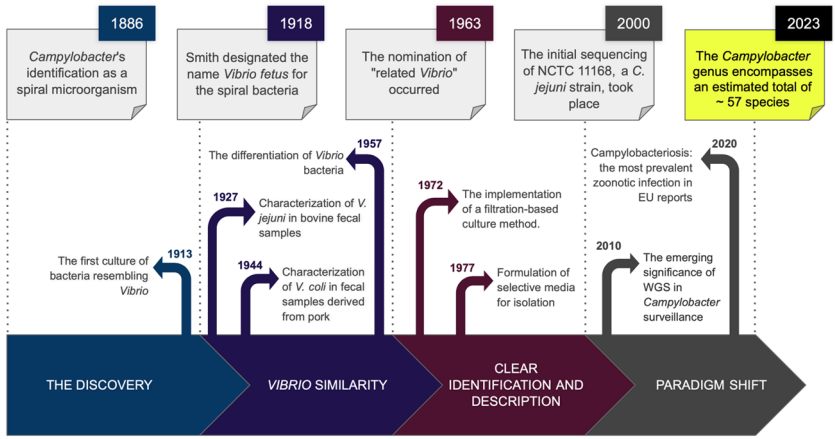

Figure 2 The taxonomy of *Campylobacter* has been shaped by a series of significant historical events.

studies estimate the economic impact of *Campylobacter* as ranging from $1.3 to $20 billion annually in the US [48]. Healthcare expenditures associated with treating Campylobacteriosis have been estimated at approximately $80 million in Canada, £51 million in the UK, and €82 million in the Netherlands [54]. In the US, *C. jejuni* and *C. coli* were responsible for 98% of reported human cases from 2015 to 2017, whilst the WHO estimates the global annual mortality rate attributed to *Campylobacter* to be approximately 37 600 [56].

The pathogen typically triggers diarroheal conditions in humans, though post-infection complications can be far-reaching, including septicaemia, hepatitis, cholecystitis, hemolytic uremic syndrome (HUS), meningitis, and even leading to foetal mortality [11, 12]. Approximately 99% of cases in humans manifest as isolated, sporadic incidents rather than forming part of recognized outbreaks, with a significant number remaining undiagnosed or unreported [3]. However, given less developed immune defences, young children from the age 0 to 5 are particularly susceptible to *Campylobacter* infections [13]. About 18% of asymptomatic school-aged children were recently discovered in one study to be carriers of *Campylobacter* species, namely *C. coli*, *C. jejuni*, and *Campylobacter lari*; of these, 84% of isolates displayed resistance to multiple drug classes [14]. Older and immunocompromised patients are also vulnerable to infection from *C. jejuni* and *C. coli* [15, 16]. The highest percentage of hospitalizations and fatalities predominantly occur amongst individuals aged 65 years and above [8]. The increasing incidence of disease in the 65 and over age group in countries such as Ireland, Australia, the UK, and Germany has been related to age-related immunosenescence and immunosuppression [27]. Evidence from acute gastroenteritis epidemiological surveillance in Australia, Canada, and the US indicates that the likelihood of experiencing severe symptoms and requiring hospitalization is greater among older people after *Campylobacter* infections [55].

A low dose of approximately 500 bacteria is capable of triggering clinical symptoms [17]. *Campylobacter* virulence factors include adhesion, chemotaxis, motility, response to oxidative stress, cytolethal distending toxin production, intricate regulation of iron uptake and invasion, lipopolysaccharide and capsular polysaccharide synthesis, and resistance to multiple drugs and bile acids [18–20]. Among the primary virulence determinants of *C. jejuni*, the cytolethal distending toxin (cdt A, B, C) complex stands out, facilitating the induction of host cell apoptosis. The bacterium's flagellum plays a crucial role in governing both motility and the secretion of invasive antigens [21]. The polysaccharide capsule (CPS) of *Campylobacter* significantly increases its virulence [22].

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2 Transmission vectors for *Campylobacter* spp. in poultry supply chains

To curb the risk of human infection, it is crucial to decrease the *Campylobacter* burden both at the farm level and during other stages in the supply chain such as the slaughtering process [29]. As an example, it has been estimated that reducing *Campylobacter* spp. levels by 3 $\log_{10}$ units in chicken caeca (part of the digestive system) at slaughter could potentially reduce the risk of human infection by up to 90% [28]. A reduction in *Campylobacter* populations present on the neck and breast skin of poultry of 10^3 CFU per gram could reduce infection risk by 50% [23–25].

Pathogenic strains of *Campylobacter* such as *C. jejuni* and *C. coli* are widely found in the intestinal ecosystems of avian species, including poultry and many wild bird species [19, 21, 25]. Domestic pets can also be a reservoir for *C. jejuni* and *Campylobacter upsaliensis* [1, 31]. Surface water contaminated with faeces from wild animals and agricultural waste provides a reservoir for diverse *Campylobacter* strains [1, 32]. Wild birds acquire *C. jejuni* from sources such as contaminated water and waste, including refuse dumps, which they can then further disseminate [1, 18]. Migratory birds significantly contribute to the distribution of *Campylobacter* within the poultry sector and the broader environment. Examination of wild birds, particularly those in close proximity to poultry farms and live bird markets, has revealed the presence of diverse, virulent, antibiotic-resistant *C. jejuni* and *C. coli* strains, [18, 21]. Waterfowl, especially geese, act as significant vectors for transmitting *Campylobacter* spp., either directly through contact with poultry or indirectly via wider environmental contamination e.g. of reservoirs supplying drinking water [18, 34, 35].

Climatic and weather conditions can also play a role in promoting the spread of *Campylobacter* spp. As an example, the emergence of campylobacteriosis as the most common bacterial gastrointestinal infection in Northern European countries such as Sweden has been associated in part with a combination of increased rainfall and higher mid-to-late summer temperatures which both encourage *Campylobacter* growth and persistence in the environment and facilitate transmission [41, 42, 44]. Other EU countries such as Ireland, Hungary, and Slovakia, as well as the UK, have been found to experience a moderate increase in cases during the early summer associated with higher temperatures and, to a lesser extent, increased precipitation [43, 45]. Increased temperatures and higher rainfall have e.g. been associated with a greater risk of contamination of water sources, resulting in higher risks for both humans and livestock [46, 47, 67].

Figure 3 illustrates a number of ways *Campylobacter* can be transmitted from the wider environment to poultry flocks. Other sources include people visiting the farm, highlighting the importance of effective biosecurity measures [25].

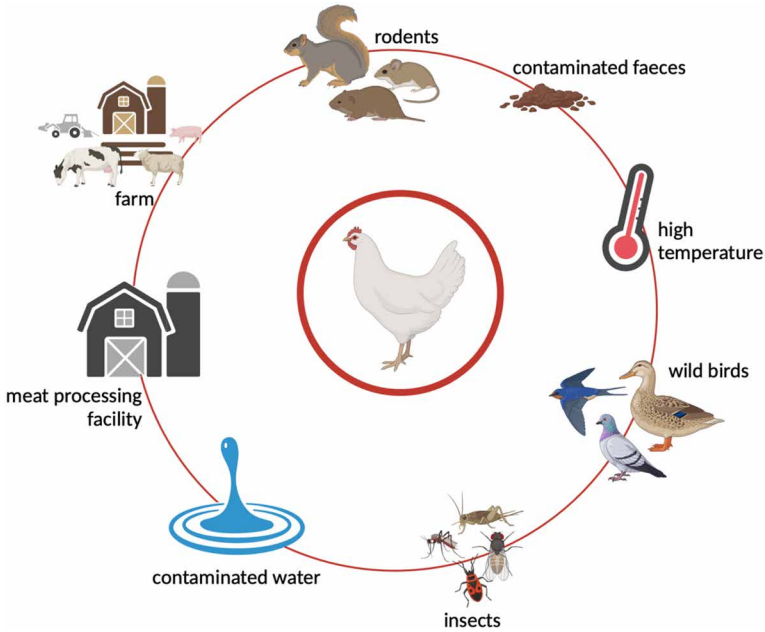

Figure 3 Potential pathways and risk factors through which *Campylobacter* contamination can occur in poultry production.

Organisms such as beetles, flies, amoebae, yeasts, and moulds have also been found to be significant vectors for the transmission of *Campylobacter* into poultry houses [2, 36]. The coexistence of *Campylobacter* cells with amoebae, yeasts, and moulds enhances pathogen survival. Microbial eukaryotes (e.g. the amoeba host *Acanthamoeba polyphaga*) can serve as environmental reservoirs of *Campylobacter* [2, 37]. Given the typical prevalence of eukaryotes in both drinking water systems and as microbial biofilms on farms, infected eukaryotes may play a pivotal role in the transmission and growth of *Campylobacter* spp. in poultry farms.

Insects can act as vectors for *Campylobacter* transmission, introducing potential contaminants into the broiler houses e.g. through poorly maintained buildings or via ventilation systems [3]. The lesser mealworm beetle and its larvae (*Alphitobius diaperunus*) e.g. have been identified as carriers of *C. jejuni* in poultry settings, with the ability to transmit *C. jejuni* not only within individual flocks but also enabling cross-contamination across successive rearing cycles. Houseflies and darkling beetles have also been identified as potential vectors for introducing *Campylobacter* to poultry flocks [36]. A study from Denmark reported a daily entry of approximately 900 flies into broiler houses, and UK research found an average of around 31 flies gaining entry every 2 h. As an example, the multi-drug resistant *C. jejuni* strain ST-1839 has been found in

numerous fly-derived isolates from turkey farms, underlining the role of insects in the propagation of resistant strains [36, 39]. Implementing fly screens could substantially reduce this contamination route [36, 38].

Campylobacter colonization in poultry farms occurs predominantly via horizontal transmission between individual birds, often mediated by feed or drinking water contaminated by *Campylobacter* present in the environment [25, 67]. Factors affecting transmission include the age of birds, flock size, type of production system, water quality, and the presence of other animals on the farm [67]. *Campylobacter* has been found to be particularly prevalent in areas such as drinking water, feed, broilers' faeces and worker's footwear rather than from sources such as litter, airborne particulates, and farm buildings [25]. *Campylobacter* exhibits remarkable endurance in farm settings, surviving in slurry, manure, and dirty water for as long as three months [25, 74]. *Campylobacter jejuni* is able e.g. to adapt to conditions of nutrient deprivation and oxygen stress [74, 75]. Moreover, survival characteristics can be boosted when in the presence of other pathogens such as *Pseudomonadaceae*, since *Campylobacter* can latch onto biofilms produced by these other pathogens [74]. Vertical transmission of *Campylobacter* from hen to egg is not a significant route [25]. Once introduced to a chicken flock, *Campylobacter* can propagate rapidly, effectively colonizing most of the avian intestinal tract – including the caeca, small intestine, and crop (part of the oesophagus) – within a week [21]. An entire flock can be colonized within 2–13 days [30].

Maternal antibodies specific to *Campylobacter* can protect the gastrointestinal tract of fledgling broilers against bacterial colonization [25]. However, this protective effect can be reduced by a restricted diet in more intensive poultry-rearing systems which can result in a less varied and competitive microbiota in the gastrointestinal tract, creating a more conducive environment for the proliferation of *Campylobacter* [2]. Contemporary developments in the field of *Campylobacter* colonization control within avian intestinal tracts have unveiled an array of novel strategies. These include the use probiotics, the incorporation of organic or inorganic acids into feed, the application of bacteriocins, the deployment of bacteriophages, and the development of targeted vaccines to boost gut and immune function and prevent *Campylobacter* becoming established [19, 32, 76–80]. A synthetic peptide, oligo-acyl-lysyl C12K-2β12, has been investigated for its ability to inhibit *Campylobacter*, particularly given its ability to withstand both heat and acid conditions characteristic of subsequent processing environments [82].

The high population density typical of commercial broiler farming also accelerates pathogen transmission from bird to bird [3]. High densities of birds in indoor settings promote higher temperatures and humidity levels which promote *Campylobacter* growth, particularly in summer months [30]. A survey of 27 poultry farms across California, Oregon, Washington, and Idaho in the US

found that farms with higher average wind speeds in the week before sampling (increasing the risk of pathogen transmission from the outside environment), combined with younger flocks (with lower immunity), higher flock densities and frequent rotations (increasing horizontal transmission) were most at risk of *Campylobacter* spp. infections [40].

3 Pre- and post-slaughter measures to prevent *Campylobacter* contamination in poultry processing

Recent model simulations highlight the benefit of combining different intervention measures against *Campylobacter* contamination. These suggest insect control as the most important measure, followed by enhanced farm sanitation and biosecurity measures to control access to the farm [29]. It was found e.g. that insect control measures such as fly screens/mesh could reduce the percentage of infected chickens from 51% to 26% and highly infected (>1000 CFU/g) chicken neck samples from 13% to 8% [3, 29]. The highest level of biosecurity can be found in modern broiler houses with minimized access points for rodents, birds, or insects, free of potential entry points such as floor, wall, or ceiling defects and with well-designed ventilation systems that prevent external contaminants from entering the poultry house [3]. Good flock management e.g. an 'all in, all out' approach where groups of birds are kept together throughout their time on the farm and segregated from other groups (rather than mixed e.g. by thinning and moving birds between flocks) is also important as are good hygiene practices such as changing clothes and footwear before entering the barn (the latter being more effective than boot dips alone) [3]. An integrated approach combining all these measures has been found to reduce the percentage of infected chickens to 5% and highly infected chicken neck samples to 2%.

Reported rates of contamination of poultry carcasses with *Campylobacter* show considerable variability worldwide with some countries (e.g. Iceland and Brazil) having historically much lower rates than countries such as the US and UK [24, 61–65]. The Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) has tracked a significant decrease in the prevalence of *Campylobacter*-positive chicken carcasses [60]. Initial figures from the 1994–1995 survey indicated a high prevalence of 88.2%. However, subsequent surveys from 2007 to 2008 and 2018 to 2019 demonstrated a substantial reduction to 46.6% and 18.3%, respectively contributing to a decline in human *Campylobacter*-associated infections [58, 60].

Results from Ireland also indicate a downward trend in *Campylobacter* prevalence in broilers, with a remarkable 45% reduction (from 98.3% to 53%) in contaminated carcasses and a 17% decrease (from 83.1% to 66%) in colonized batches of poultry. Despite this progress, the incidence of *Campylobacter* in

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humans has not seen a corresponding decline, with confirmed cases increasing from 1752 in 2008 to over 3000 in 2018. Consequently, poultry meat remains a significant source of *Campylobacter* contamination in Ireland [61]. There has been a similar trend in New Zealand where improved control measures have resulted in a 74% decrease in poultry-attributed campylobacteriosis cases [58] though without eradicating disease outbreaks related to the pathogen [66]. In the Canadian poultry sector, *Campylobacter* has been found to be present in about 24% of broiler chicken samples at the moment of slaughter, 33% of poultry samples during the processing stage, and up 42% of chicken samples available for retail sale, indicating the challenges and importance of controlling the pathogen at each stage of the supply chain, including proper handling, storage, and cooking of poultry meat [8, 67, 71].

The pre-slaughter transport phase entails moving broilers from the farm to the abattoir. Birds are placed into cages for transport, denied feed and water, and subjected to stress during travel, promoting cross-contamination from a single colonized broiler to others via contact with faeces or feathers [25]. The stress broilers experience during transportation increases the excretion of faecal matter which promotes contamination [2]. Measures such as cleaning and sanitizing contaminated crates before and after broiler transportation may have only a modest impact in preventing *Campylobacter* infection [70, 73].

Defeathering, trimming, and deboning can inadvertently foster an environment conducive to cross-contamination of *Campylobacter* species, rendering previously uncontaminated carcasses vulnerable [72]. De-feathering, evisceration, and portioning result in intestinal contents being expelled or leaked, releasing contaminated material particularly from the crop and the intestines [12]. Deboning can lead to bacterial proliferation on both equipment and within the slaughterhouse environment [72]. *Campylobacter* spp. has been found particularly on de-feathering and evisceration equipment, workers' gloves, shackles for holding carcasses, and conveyor belt equipment [25].

As many other pathogens, *C. jejuni* has shown a remarkable ability to attach to an array of materials such as glass, wood, stainless steel, or plastic, forming a biofilm that is resistant to cleaning [12]. *C. jejuni* e.g. adheres strongly to stainless steel interfaces, even in nutrient-deprived conditions and at temperatures of 20°C [73]. The use of antimicrobials and quorum sensing (QS) signal inhibitors can eradicate biofilms, though this is a risk of bacterial resistance developing through mechanisms such as horizontal gene transfer and spontaneous mutations [54]. Disinfectants such as sodium hypochlorite (NaOCl) are not always effective in eradicating pathogens tenaciously adhering to carcasses [12].

Ways of preventing or removing bacterial contamination of carcasses can be grouped into three categories [81]:

- physical interventions (hot water, cold plasma, steam irradiation, ultrasound, ultraviolet light, air chilling, freezing, and others);
- chemical interventions (natural antimicrobials, EOs, organic acids (e.g. lactic acid), chlorine, hypochlorite, electrolysed oxidizing water, and ozonated water); and
- biological interventions (bacteriophages and probiotics).

The application of 2% lactic acid on poultry carcasses can potentially lower the infection risk of *Campylobacter* by between 37% and 56% [24]. Employing acidified sodium chlorite (1200 mg/L) and trisodium phosphate (10–12%, at pH 12) in carcass treatment can diminish this risk by 75–96% and 67–84% [24]. Techniques such as the use of peracetic acid and water immersion chilling, a widely used procedure in the US, also contribute to pathogen reduction strategies [60].

4 The use of bacteriophages to control *Campylobacter* in the poultry gut

Bacteriophages, viruses targeting bacteria, and bacteriocins, antimicrobial peptides produced by probiotic lactic acid bacteria, have emerged as potential feed additives to suppress *C. jejuni* populations in the caecum of broilers [3, 25]. *Campylobacter* isolated and cultivated phages have been explored to control *Campylobacter* colonization within the gastrointestinal tract of live birds [80]. This strategy has also been extended to poultry carcasses to lower *Campylobacter* levels during processing. Phage therapy maintains the health of the broilers' intestinal microbiota while effectively tackling the pathogen [80]. Most phage research focuses on the *Myoviridae* family of *Campylobacter* phages, while studies involving the *Siphoviridae* family are comparatively uncommon [85]. In analysing the connections between phages and antibiotic-resistance genes, it was found that the development of phage resistance often coincided with heightened antibiotic sensitivity in bacteria [86]. This complex interaction suggests the potential for phage resistance to mitigate antibiotic resistance. Bacteriophage treatment has been shown to decrease caecal levels of *C. jejuni* in broiler flocks by approximately 5 logs CFU/g [87]. However, disadvantages include their low dilution rate in the gut [2].

Studies on broilers have shown that the deployment of bacteriophages (including but not limited to NCTC 12671, 12669, 12672, 12673, 12674, 12678, 12684, CAM-P21, Cj6, CP8, CP34, CP81, phiCcolIBB35,phiCcolIBB12, and phiCcolIBB37) can appreciably reduce *Campylobacter* levels when administered 24–48 h before slaughter [24, 25, 80]. The introduction of individual bacteriophages or their combinations (like phage NCTC 12673, 12674, and 12678) has resulted in notable reductions of bacterial logarithmic

counts in caecal content, ranging between 2.5 and 3.2 CFU/g. It was also shown that phage cocktail treatments (CP20 and CP30A), unlike broad-spectrum antibiotics, do not adversely affect the gut microbiota of broilers, thus preventing dysbiosis [25].

H-fiber, sourced from a prophage akin to *C. jejuni* Integrated Elements (CJIEs)-1, is a distinct receptor-binding protein [80]. Zampara et al. developed innolysins to specifically target *C. jejuni* by fusing this H-fiber with T5 endolysin [88]. The application of these innolysins, which merged the enzymatic function of endolysins with the binding ability of phage receptor proteins (in order to amplify their effect on Gram-negative bacteria), led to a 1.18 to 1.63 log reduction in *C. jejuni* CAMSA2147 on chicken skin refrigerated to 5°C [24, 88].

Richards et al. showed how a CP30A and CP20 phage cocktail significantly reduced intestinal *C. jejuni* populations in colonized birds over 5 days without impacting the diversity of caecal and ileal microbiota [89]. An evident effect was observed in the caeca, with bacterial counts reduced by 2.4 $\log_{10}$ CFU/g. Significant bacterial reductions were found in the ileum (1.36 $\log_{10}$ CFU/g) on day 2 and the colon (1.74 $\log_{10}$ CFU/g) on day 3. Both phages were detected in all three compartments of the chicken's gastrointestinal tract for 5 days, implying successful *in vivo* replication and coexistence without competition [89]. Whilst completely eradicating *Campylobacter* in the bird's gastrointestinal tract with phage therapy may be unachievable, these reductions are significant [80].

Others have identified CAM-P21, a broad-spectrum phage, exhibiting superior performance under varied stress conditions and a higher titer than *Myoviridae* family phages [85]. CAM-P21 significantly decreased viable *C. coli* counts by more than 2 logs after incubation at 42°C and 37°C *in vitro* over 12–24 h. In another study, oral administration of two field phages to experimentally infected broiler chickens aged 37 days significantly diminished Campylobacter levels by 2 $\log_{10}$ CFU/g at 40 days [24].

Companies such as PhageBiotech (Israel), Micreos (Netherlands), and Initralytix (USA) are developing commercially available phage-based products aiming to control pathogens including *Listeria*, *E. coli*, *Campylobacter*, *Salmonella*, and other species. Phage therapy can be improved in terms of antimicrobial precision and efficiency via CRISPR-Cas systems as demonstrated in an *in vivo* study by reducing *Clostridioides difficile* infection [90].

5 The use of probiotics in poultry feed

Modification of gut microbiota through competitive exclusion using probiotics and faecal microbial transplants (FMTs) has demonstrated remarkable potential in mitigating *Campylobacter* colonization [24]. Introducing a non-pathogenic strain into a niche occupied by a pathogenic *Campylobacter* strain can

outcompete the latter, leading to its removal from the niche [28]. This strategy is often adopted with innovative probiotic species or strains to regulate pathogens such as *C. jejuni*.

Probiotic mixtures containing *L. paracasei* J.R, *L. rhamnosus* 15b, *L. lactis* Y, and *L. lactis*, have been found to result in as much as 5 $\log_{10}$ CFU/mL reduction in caecal *Campylobacter* counts [91]. Equally impressive outcomes were reported where the caecal load of *C. jejuni* in broiler chickens was decreased by up to 6 $\log_{10}$ [92]. This substantial reduction was achieved by administering a probiotic mixture comprising *Bifidobacterium animalis*, *Enterococcus faecium*, *L. salivarius*, *L. reuteri*, and *Pediococcus acidilactici*.

Partial success has been recently observed with the use of lactic acid bacteria [54]. Incorporating acidic feed additives and acids into drinking water has shown promising outcomes. Including the probiotic (Lavipan) in broiler chicken feed significantly mitigated invasion of *Campylobacter* spp. within the birds' gastrointestinal tract [93]. Regular oral intake of *L. salivarius* SMXD51 every 2–3 days from day 1 to day 35 of age can diminish caecal *Campylobacter* counts by 0.8 $\log_{10}$ at 14 days, with a more significant reduction of 2.8 $\log_{10}$ noted at 35 days [94]. The probiotic *E. coli Nissle* 1917 (EcN), either unencapsulated or encapsulated within chitosan-alginate microspheres, has been administered orally to chickens three times weekly for 2 weeks [95]. The authors observed a reduction in caecal *C. jejuni* colonization between 2 and 2.5 log CFU/g. The treatment also enhanced the chickens' growth performance, intestinal structure, and immunity without disrupting the gut microbiota [95]. EcN is a compelling case of a highly effective probiotic, distinguished by its unique Lipopolysaccharide (LPS) that invokes immunogenic responses without inducing immunotoxic effects [77]. The biological safety profile of EcN has been rigorously evaluated in animal studies and human trials [77].

Šimunović and collaborators have shown that *B. subtilis* PS-216 exhibited an inhibitory effect on *C. jejuni* growth, with a substantial reduction in sterile chicken litter (by 4.07 log) and in sterile intestinal content (by 2.26 log) [28]. *B. subtilis* PS-216 can proliferate even in nonsterile intestinal experimental conditions, suggesting its potential utility as a chicken probiotic that can be assimilated into the avian intestinal microbiota. *B. subtilis* PS-216 manifested the most potent antagonistic effect against *C. jejuni* NCTC 11168 under conditions mimicking those of the chicken's environment, specifically, 42°C, microaerobic atmosphere, and chicken litter medium [28]. *Lactobacillus gallinarum* PL53 (at a concentration of 10^8 CFU) has been found to diminish *C. jejuni* presence within the cloaca and caeca, which reinforces the potential of employing such microorganisms as suppressors of *Campylobacter* colonization during primary production [77, 96].

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6 The use of chemical agents for decontamination

The poultry industry routinely employs chemical decontamination methods to remove contaminants from carcasses. These methods involve using substances interchangeably referred to as sanitizers, disinfectants, sterilants, antiseptics, biocides, antimicrobials, or decontaminating agents [97]. In the US, the deployment of such processing aids is regulated and monitored by the Food and Drug Administration (FDA) as well as the USDA [97]. In contrast to the multiple chemical decontamination strategies permitted in the US, the EU has shown a more limited approach, currently only authorizing up to 5% lactic acid as a decontaminant [81]. Various compounds – including acetic, citric, peracetic, peroxyacetic, and lactic acids, along with acidified sodium chlorite, cetylpyridinium chloride, chlorine, vinegar, sodium hypochlorite, and trisodium phosphate – can contribute significantly to the reduction of *Campylobacter* spp. on poultry carcasses during the slaughter process [83, 84, 97, 98].

According to a recent meta-analysis, cetylpyridinium chloride, acidified sodium chlorite, and trisodium phosphate have demonstrated the most significant log reduction estimates ≈1.38 and 1.26 $\log_{10}$ CFU, respectively [97]. In contrast, treatments involving cetylpyridinium chloride and a blend of potassium hydroxide and lauric acid revealed lower reductions. A combination of ethanol, acidic calcium sulfate, lactic acid, propylene glycol, and sodium dodecyl sulphate resulted in an impressive log reduction estimate of ≈ 4.91 $\log_{10}$ CFU [97]. There is considerable interest in the commercial antimicrobial product Amplon, a blend of sulfuric acid and sodium sulfate [48]. The USDA-FSIS has allowed this product for spray, wash, and dip treatments. A recent investigation demonstrated a significant *Campylobacter* reduction, averaging a decrease of ≈ 1.53 log CFU per chicken, following a 15-s post-chill immersion in an Amplon solution [48].

Peracetic acid (PAA) is routinely employed in the US poultry processing industry, with concentrations typically between 200 and 2000 ppm during chilling, dependent on the duration of chilling [99]. This approach has demonstrated considerable efficacy in mitigating *Campylobacter* numbers in chicken carcasses, showing reductions ranging from ≈ 1.5 to ≈ 2.0 $\log_{10}$ CFU/g or ≈1.8 to 2.6 $\log_{10}$ CFU/mL in microbial load and a 50–100% decrease in prevalence [100]. Notably, no discernible negative impact on chicken quality was reported, with sensory evaluations indicating that 1200 ppm of PAA not only resulted in a larger *Campylobacter* reduction (≈2 $\log_{10}$ CFU/g) compared to 50 ppm chlorine but also maintained the sensory quality of the chicken. PAA has proven highly effective in post-chill dip treatments applied to chicken breasts [48]. One investigation reported reductions of 2.23 and 4.08 log CFU/chicken in *Campylobacter* and *Salmonella* following a 15-s immersion in a solution containing 750 ppm PAA [48].

Proteomic analysis indicates that *C. jejuni* strain 2704 rapidly reacts to PAA stress, with initial response occurring within 15 min and full adaptation between 30 and 45 min [100]. This response manifests as perturbations in the cytoplasmic redox state and central metabolism. Regulatory and homeostatic responses, coupled with adjustments to energy metabolism and flagellar/chemotaxis protein response, underpin *C. jejuni* survival under PAA exposure. Optimizing PAA application necessitates understanding how to bypass these adaptive responses for optimal reduction efficacy. Certain heat stress proteins (Lon, HtrA, HslR) suggest that heat stress response may offer cross-protection when *Campylobacter* is later exposed to oxidative PAA treatment [100].

7 The use of natural antimicrobials

Mixtures of natural antimicrobials, including components like organic acids and plant extracts, reduce the colonization or infection of epithelial [101–108]. Research has investigated the efficacy of a blend of natural antimicrobials (A3001) in limiting the adherence of T6SS$^{+/-}$ *C. coli* isolates (NC1 $^{hcp-}$, NC2 $^{hcp-}$, and NC3 $^{hcp+}$) to chicken skin and carcasses [19]. The antimicrobial mixture significantly diminished the colonization capacity of *C. coli* isolates on chicken skin and carcasses, resulting in an approximate 3-log reduction [19]. Following an *in vitro* infection model, a 0.5% mixture concentration led to significant reductions of adherence in the HCT-8 cell line and downregulated the *hcp* gene in the NC3 isolate, which indicates the presence of T6SS [19].

Olive leaf extract (E1), notably rich in oleuropein – a precursor to hydroxytyrosol, exhibits a significant capacity to inhibit the proliferation of *Campylobacter* [109]. E1 has potent antibacterial activity at a MIC range of 0.1–2 mg/mL, curtailing the growth of *C. jejuni* strains from 4.12 to 8.14 log CFU/mL while inducing a reduction between 6.99 and 7.99 log CFU/mL against *C. coli* strains [109].

Multi-omics analysis and chicken trials have shown that *C. jejuni* colonization notably increases levels of CREG-1, creatinine, and 3-[2-(3-Hydroxyphenyl) ethyl]-5-methoxyphenyl, while concurrently decreasing sphingosine, SP d18:1, high mobility group protein B3, and several phosphatidylcholines (PC P-20:0_16:0, PC 11:0_26:1, and PC 13:0_26:2) [110]. These essential proteins and lipids are implicated in inflammation and immunity in *C. jejuni*-colonized birds. The authors observed average colonization of *C. jejuni* for control groups showing positive results at approximately 6.5 log CFU/g of caecal content on day 14. Treating with an in-water supplement of 0.125% eugenol nanoemulsion (EGNE) reduced counts by around 2.5 log CFU/g compared to the positive controls. Additionally, they detected a rise in the blood serum concentrations of various metabolites, such as 5-hydroxyindole-3-acetic acid, pyridoxal, taurine, indole-3-lactic acid, monoolein stachydrine, taurousodeoxycholic

acid, and methionine sulfoxide, after EGNE treatment [110]. Collectively, these metabolites appear to promote favourable responses in broilers through their antioxidant, anti-inflammatory, and antimicrobial properties [110].

According to *in vitro* studies, oregano species and essential oils (Eos) derived from them are rich in antimicrobial bioactive compounds such as carvacrol, p-cymene, thymol, and g-terpinene and are known to exhibit anti-campylobacter activity [111]. When EO derived from *Origanum compactum* was combined with tetracycline or ampicillin, more efficient inhibitory synergy effects were noted against multidrug-resistant and strong biofilm *Campylobacter* strains [111, 112]. Research simulating industrial environments has shown that a significant decrease in *C. jejuni* counts on chicken skin can be achieved – approximately 2.4–4 log10 CFU/sample [112].

Bio-responsive composite liposomes imbued with the EO from *Litsea cubeba* have exhibited impressive antibacterial activity [113]. With an inhibition zone of 23.5 mm, just 20% (v/v) of these composite liposomes can neutralize 99% of *C. jejuni*. After spraying, these liposomes demonstrated a prolonged capacity to impede bacterial proliferation for up to 5 days on refrigerated chicken meat inoculated with *C. jejuni*. The authors reported that these liposomes did not affect the food's colour and texture and could pave the way for the use of liposomes as potent antimicrobial agents [113].

Other research has indicated that edible film, derived from chitosan and pectin and incorporating 2% eugenol, can decrease *C. jejuni* loads by 2 and 3 log CFU/sample in chicken wingettes stored at 4C° for 7 days [49]. A combination of carvacrol or eugenol with chitosan considerably suppresses the expression of specific genes tied to motility (*motA*), stress response (*katA*), and quorum sensing (*luxS*) in *C. jejuni*, which may inhibit the bacteria's survival in poultry [49, 114].

Edible coatings derived from natural plant-based sources and enriched with natural antimicrobials are emerging as innovative solutions for enhancing product longevity and quality [97]. One study comprising 60 trials analysed the influence of eugenol-strengthened pectin and chitosan coatings [97]. The collective alteration in concentration was projected at 2.36 $\log_{10}$ CFU declines within a 95% CI, and significant heterogeneity was observed (I2 = 88%, $P <$ o.001) [97]. Carvacrol-infused chitosan and gum arabic films were evaluated across 69 trials from two studies, generating a cumulative effect estimated at 2.00 $\log_{10}$ CFU reductions and similar significant heterogeneity [97].

8 Physical decontamination treatments

Physical decontamination strategies involve measures to lower contamination in broiler carcasses or meat products by manipulating temperature or applying electrochemical, electromagnetic, or mechanical processes that disrupt the

integrity of pathogenic cells [97]. These interventions include crust freezing, immersion chilling, forced air chilling, hot water treatment, vent plug application, rapid surface cooling, and steam-ultrasound treatment [98]. A total eradication of campylobacteriosis risk can be achieved via industrial-scale radiation or the cooking of poultry meat post-slaughter [54]. Compared to chemical methods, physical decontamination has a greater impact on prevalence, while chemical treatments more significantly affect bacterial concentration [98]. Decontamination interventions applied through immersion exhibit greater effectiveness than those executed via spraying. While it has yet to be utilized in commercial contexts, deploying cold plasma technology is an innovative and promising technique, with its potential even extending to pre-packaged food items [48].

Steam pasteurization has recently emerged as a promising approach for surface decontaminating broiler carcasses [115]. This technique entails the brief application of saturated pressurised steam. A pilot-scale steam decontamination system (Deconizer) has proved efficacious in diminishing *Salmonella* and *Campylobacter* [116]. The decontamination effect ranged from a 1.22 to 3.33 log reduction for aerobic bacteria, a 1.36 to 3.05 log reduction for *Salmonella Enteritidis*, and a remarkable 0.84 to 4.32 log reduction for *C. jejuni*. Treatment for 5 s led to greater microbial reductions than 3-s treatments, observed at 95°C and 120°C. This effect was more pronounced on the breast portion than on the legs and wings [116].

Freezing poultry carcasses provides a reliable way to achieve a two-log decrease in *Campylobacter* logarithmic counts [3]. In Iceland, a requirement that broilers processed from *Campylobacter*-infected flocks be frozen had a notable effect, significantly diminishing the incidence of *Campylobacter*-induced enteritis in humans [3]. A reduction of over 90% in the risk can be attained by freezing carcasses for a period of 2–3 weeks [54]. Risk reduction ranging between 50% and 90% is possible through a combination of 2–3 days of freezing, succeeded by warm water or chemical carcass decontamination [54]. Freezing can diminish *Campylobacter* counts, but traces of viable bacteria can still be detected on poultry even after weeks of freezing at −20°C [3]. Strain variability and environmental pressures can influence survival potential and the ability to colonize hosts. Despite reducing culturable bacteria during freezing, research indicates that these organisms can maintain viability over extended periods in food stored at low or freezing temperatures [12]. The food industry's use of cold chains for product freshness can facilitate the survival of *C. jejuni* and *C. coli* in meat [12, 19]. The potency to enter a viable but non-culturable state (VBNC) may underpin this pathogen's adaptive response to environmental stress [12, 19].

Crust freezing involves rapidly freezing the carcass surface while leaving the underlying meat unfrozen. However, its effectiveness was substantially

lower in real conditions before-after experimental trials compared to controlled challenge trials, suggesting that the practicality of its large-scale implementation remains to be determined [97]. Techniques such as scalding and chilling can efficiently diminish pathogens' prevalence and concentration, but their efficacy could be enhanced by incorporating processing aids to mitigate potential cross-contamination between carcasses and faecal contamination [97]. Physical decontamination methods have a more pronounced effect on reducing *Campylobacter* prevalence, decreasing relative risk by approximately ≈68.7%, as compared to a ≈30.3% reduction achieved through chemical treatment [98]. Laboratory before-after trials reported the effects of brief (up to 40 s) hot water immersion at 70–80°C [97]. The collective log reduction from these challenge trials indicated a ≈1.23 $\log_{10}$ CFU decrease. An assessment of in-plant decontamination of broiler carcasses from Norway has concluded that broiler carcass immersion for 6 s at 80°C was the most effective hot-water treatment by causing significant declines of the *E. coli* by 1.1 log CFU/mL and without causing any skin damage [117]. Consequently, while hot water immersion may show moderate efficacy in conditions of high experimental inoculation, its efficiency could be restricted under processing plant conditions with natural contamination [97].

Decontamination using immersion can be effective, resulting in the reduction of ≈ 0.9 $\log_{10}$ CFU/carcass compared to spraying, which results in a ≈0.5 $\log_{10}$ CFU/carcass reduction [97]. A meta-analysis employing a random-effects model found a significant reduction in *Campylobacter* concentration, with a pooled decrease of 0.6 $\log_{10}$ CFU/carcass and a ≈ 57.2% decline in relative risk of *Campylobacter* spp. prevalence in broiler carcasses. Using 0.1% PAA, applied through immersion and electrostatic spray methods to chicken wings, yielded an average *Campylobacter* reduction above ≈ 2.1 logs CFU/g [48].

9 Developing effective vaccines

Vaccination is still expensive, highly specific, and difficult to design against *Campylobacter*. Even though efforts have been underway for over 20 years to formulate an effective *Campylobacter* vaccine for broilers, commercial success remains elusive [24, 53, 118]. Traditional approaches towards developing a vaccine against *C. jejuni* in poultry have yet to yield a successful result that effectively stimulates immune response and combats bacterial colonization in the poultry's caeca. Given the absence of an effective vaccine and the alarming rise of antimicrobial-resistant strains, there is an urgent need to pinpoint novel intervention targets [53].

Genes essential for the growth and survival of *Campylobacter* present appealing prospects for these interventions. The diversity of genes in

Campylobacter highlights numerous indispensable factors critical to the fitness and survival of pathogen, many of which currently bear an unknown or hypothetical function [114, 119]. Deciphering these genes' roles will greatly enrich our understanding of *Campylobacter*'s fundamental biology, enabling innovative intervention strategies [114, 119]. Computational *in silico* prediction and gene expression analysis could potentially unveil promising vaccine candidates against *C. jejuni* [53]. Reverse vaccinology could pave the way for *C. jejuni* vaccine development. This novel strategy swiftly identifies promising antigenic candidates through microbial genome sequencing [53]. However, despite its ability to streamline potential vaccine candidates, the immediate prospect of yielding a successful vaccine remains modest.

In developing vaccines for broilers, several crucial factors must be taken into account [3, 53, 118]:

- the presence of multiple *C. jejuni* genotypes;
- the short lifespan of broilers;
- the immunological immaturity of the birds at vaccination;
- the potential interference of maternally derived antibodies;
- the necessity for effective antigen presentation at the gut mucosal surface;
- the lack of suitable avian mucosal adjuvants;
- the convenience of the vaccination method e.g. oral or in ovo; and
- the overall cost of the vaccine.

Three responses should be taken into account during vaccine manufacture [118, 120]:

- the influence of maternal immunity;
- initiation of innate immune responses, characterized by the involvement of Toll-like receptors, chemokines, and antimicrobial b-defensin peptides; and
- activation of adaptive immune responses, which induce the production of antibodies or cytokines.

Recent recombinant vaccines targeting *Campylobacter* colonization in broilers have shown significant reductions in Campylobacter colonization, up to ≈2 $\log_{10}$, following the administration of FlaA, FlpA, or a fused protein comprising CaDF-FlaA-FlpA [3, 24]. A ≈ 2.5 $\log_{10}$ decrease in *Campylobacter* colonization was achieved using a recombinant attenuated *Salmonella enterica* to express the Dsp antigen [121].

The immune system and microbiota experience co-evolution in the chicken gut. Gloanec et al. 2022 have recently reported a minor yet significant decrease in caecal *Campylobacter* counts in the DNA prime/protein boost flagellin

vaccinated group, a finding further supported by microbiota analysis which showed a differential association of *Campylobacter* genus with the placebo group [122]. The vaccinated group exhibited temporarily heightened levels of Interleukin-10, whereas the placebo group displayed an elevated gene expression of the antimicrobial peptide avian β-defensin-10. The vaccination strategy resulted in a modest reduction in Campylobacter burden, with a mean difference of 1.3 $\log_{10}$ CFU/g noted on day 40 [118]. Moreover, the impact of vaccination on the structure and composition of the caecal microbiota was evident, with distinct augmentations in operational taxonomic units identified as *Ruminococcaceae* and *Bacillaceae* on the 40th day [122].

In-ovo vaccination of embryonated chicken eggs at 18 days with Gram-positive Enhancer Matrix (GEM) particles containing dual *Campylobacter* antigens (CjaA and CjaD) resulted in a modest reduction ≈1 $\log_{10}$ in caecal colonization by *Campylobacter* in broiler chickens aged 3 and 4 weeks post heterologous *C. jejuni* challenge [24]. Interestingly, when these antigens were encapsulated within a liposome, a greater decrease in *Campylobacter* count by 2 $\log_{10}$ was observed [24]. While the reduction in *Campylobacter* was moderately successful, these findings suggest the potential efficacy of *in-ovo* immunization, a strategy that could be augmented with booster vaccinations after hatching. Applying nanoparticle-based vaccines via oral and *in-ovo* routes has garnered interest among several research groups [123, 124]. Vaccinating broiler chickens with *C. jejuni* lysate and a Toll-Like Receptor-21 ligand (CpG ODN 2007) encapsulated within poly-lactic-co-glycolic acid nanoparticles stimulated innate mucosal responses in both the intestine and caecal tonsils, amplified the serum anti-*C. jejuni* IgY antibody titers altered the caecal microbiota composition and diminished the caecal *C. jejuni* count by ≈ 2.4 $\log_{10}$ in the vaccinated broiler chickens [123, 124].

Upcoming research should focus on discovering unique, highly conserved immunogenic proteins that can stimulate cross-protective immunity against diverse *Campylobacters*. The inventive deployment of emerging technologies, such as reverse vaccinology, could facilitate the prediction of new antigenic targets, potentially leading to the fabrication of a multi-epitope vaccine offering broad-spectrum protection against varied *Campylobacter* strains [24, 53]. Subsequently, this could drive the advancement of delivery systems designed for targeted administration of vaccine compositions to *Campylobacter* colonization sites in chickens.

10 Conclusion

Addressing the challenge of *Campylobacter* in poultry is a multifaceted endeavour that necessitates a comprehensive approach. The interventions discussed in this chapter clearly point out both the challenges and also the opportunities.

Biosecurity stands clearly as the first line of defence. A farm's biosecurity strategy is as much about the barriers it builds against external threats as the internal protocols it upholds. Cleanliness, limiting external exposure, including rigorous pest management, could help minimize the presence of *Campylobacter*.

The feed and water interventions including natural antimicrobials, organic acids, and essential oils have produced variable results, indicating that a better understanding of their mechanisms of action is required. Our journey takes us through the maze of the avian gut, a bustling metropolis of microbes, where probiotics and competitive exclusion principles come into play. This is nature's own battleground, where microbial alliances can be leveraged to keep *Campylobacter* at bay. Nevertheless, as with all natural systems, predicting outcomes becomes as much an art as a science. Bacteriophages and their role have also been described. Their precision in targeting specific bacterial strains paints a promising picture, but the storyline is still unfolding, and the results remain to be seen.

Chemical and biological interventions in a world growing increasingly wary of antibiotic resistance are also required. Physical interventions, such as steam, ultraviolet light, and chilling, are also efficient. Often seen in the post-harvest stages, these established technologies underscore the importance of integrating strategies for a holistic defence. And then there is the elusive quest for the perfect vaccine. This is a journey punctuated with highs and lows, the narrative enriched by novel methodologies, from reverse vaccinology to nanoparticle delivery mechanisms. Every twist, turn, setback, and breakthrough brings us closer to that dream of comprehensive protection.

One truth emerges in piecing together these narratives: the fight against *Campylobacter* is multifaceted, demanding many strategies. It is not about choosing one path but understanding how each can be interwoven to craft a holistic approach. Our story, thus, is not just about tackling a bacterial adversary but also about human resilience, ingenuity, and the never-ending pursuit of knowledge. It is evident that the story of *Campylobacter* interventions is far from over, but with every stride, we move closer to a safer, healthier future for livestock and humans alike.

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