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CHAPTER 5
Role of Pre, Pro and Synbiotics in Reducing Zoonotic Pathogen Abundance, Lowering Antimicrobial Resistance and Improving Food Safety: Old Concepts with a New Perspective

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Abstract

Zoonotic diseases are a multifaceted beast whether bacterial, viral or fungal; they spread to people through contact with animals carrying the pathogen. An estimated 60% of human infections and at least 75% of emerging diseases are zoonotic. The World Health Organization and the World Organisation for Animal Health agree that controlling zoonoses at their animal source is the most effective and economical way of protecting public health. Since the removal of antibiotics at the sub-therapeutic level, several strategies envisaging the use of probiotics and prebiotics, or a combination of them (i.e. synbiotics), as feed additives in poultry nutrition are gaining popularity. They are found to be effective in maintaining resilient performance without medication, consequently, a key subject that led to the optimization of different types of strategies, showing increased zootechnical performance, reduced pathogen growth and maintaining animal welfare. Nevertheless, while there is emerging evidence that probiotics can help reduce foodborne pathogens, studies link this approach to antimicrobial resistance rate owing to selection pressure, gene reservoir mechanisms and horizontal gene transfer. The main genus of interest in this chapter is *Campylobacter*. The aim is to review the existing literature, focusing on the application of non-antibiotic nutritional strategies in poultry,

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including probiotics or prebiotics administered alone or in combination, cell-free supernatant, bacteriophage therapy and other non-organic molecules such as peracetic acid, to combat pathogen colonization pre-slaughter. While using established disease challenge models, the mode of action of existing probiotics can be broadly described in three ways, including, but not limited to, competitive exclusion, antagonism and stimulation of the host immune response. Although prebiotics are relatively easy to add to feeds, there are many obstacles to their utilization, mainly due to in-feed stability and viability when delivered through the gastrointestinal tract to trigger a visible response both at the microbiota and host level. European studies indicate that on-farm non-antibiotic interventions could effectively lead to a 1.0 $\log_{10}$ *Campylobacter* reduction in caeca/faecal/carcass of chickens, resulting in a 90% reduction in human infections, but the biological significance of this reduction in *Campylobacter* concentration and the etiopathogenesis of related zoonoses remain a question. We will provide historic perspective and recent updates on current non-antibiotic approaches and their impact on *Campylobacter* presence associated with poultry, with the main interest in their impact on poultry production and human health.

5.1 Introduction

Zoonosis is an infectious disease of humans caused by pathogens (e.g. bacteria, viruses) of animal origin, either through direct contact or contaminated food, water or the environment. The World Health Organization estimates zoonosis incurrence in almost one in ten people (600 million), after eating contaminated food, resulting in a global annual burden of 33 million disability-adjusted life years (DALYs) and 420,000 premature deaths (WHO, 2015). Additionally, food supply globalization leads to an increased risk factor worldwide, including antimicrobial resistance (AMR) linked with foodborne pathogens because of the inappropriate use of antimicrobials in humans, animals and plants. It is estimated that by 2050, 10 million lives will be at risk, and a cumulative US$100 trillion will be lost owing to the impact of AMR if no proactive solutions are taken (O'Neill, 2016; WHO, 2022). The most reported zoonoses in humans are campylobacteriosis and salmonellosis (EU, 2021). *Campylobacter* is a fastidious commensal organism routinely found in cattle, sheep, swine and poultry, which can survive in various environments, yet broiler meat is considered the most common host (Hermans *et al.*, 2012; Sasaki *et al.*, 2013). The epidemiological evidence indicates that *Campylobacter*-positive undercooked chicken and poultry products such as contaminated chicken liver are trivial sources of human infection (Mor-Mur and Yuste, 2010). However, the only recorded campylobacteriosis cases currently reported are nosocomial, indicating that the reported figures are the tip of the iceberg.

5.2 Avian Gastrointestinal Microbiota and Microbiome

The terms 'microbiota' and 'microbiome' are often interchangeable but they are not perfect synonyms. 'Microbiota' refers to bacteria, fungi, archaea, protozoa, and

viruses living in the gut, whereas the term 'microbiome' refers to the collective genomes of the microorganisms in a given environment (Marchesi and Ravel, 2015). Nutritional studies in poultry have shown clear connections between microbiota and host growth, showing that diet is the main driver in shaping microbial communities over time (Borda-Molina *et al.*, 2018). Gut communities have both benefits and costs to the host, with the primary benefits being the competitive exclusion of pathogens, immune stimulation and programming, and contributions to host nutrition (Dibner and Richards, 2005). In the distal gut (i.e. ceca and colon), the microbiota also produces energy and nutrients such as vitamins, amino acids and short-chain fatty acids (SCFAs) from the undigested feed, which eventually become available for the host (Gaskins *et al.*, 2002). SCFAs also play a significant role in regulating gut health; indeed, enterocytes use mostly butyrate for energy production, which also has anti-inflammatory properties (Hamer *et al.*, 2008). SCFAs also impede the invasion and colonization of pathogens by lowering gut pH and inhibiting the conversion of bile to secondary bile products (Christl *et al.*, 1997; Ricke, 2003).

The chicken's gastrointestinal tract includes the crop, proventriculus, gizzard, duodenum, jejunum, ileum, caeca, colon and cloaca (Yeoman *et al.*, 2012). Each section has different metabolic functions that shape the microbial community and vice versa. The chicken crop harbours 10^8 to 10^9 cfu/g bacteria, which is usually dominated by lactobacilli (Gong *et al.*, 2007). Most bacteria in the gizzard (10^7–10^8/g) are *Lactobacillus*, *Enterococcus*, lactose-negative enterobacteria and coliform bacteria (Rehman *et al.*, 2007). Among the small intestinal segments, the bacterial density is the lowest in the duodenum due to short passage time and a dilution of digesta by secreted bile (Shapiro and Sarles, 1949). The duodenal bacterial community mainly consists of clostridia, streptococci, enterobacteria and lactobacilli (Waite and Taylor, 2015). *Lactobacillus* dominates the ileum microbiota as the primary group (70%), followed by members of the family *Clostridiaceae* (11%), *Streptococcus* (6.5%) and *Enterococcus* (6.5%) (Lu *et al.*, 2003). Compared to the ileum, the cecum has a more diversified and stable microbial community, reported to be around 10^{10}–10^{11}/g of bacteria, including anaerobes (Videnska *et al.*, 2013).

The chicken gut microbiota changes from crop to colon, and these changes are affected by diet (Torok *et al.*, 2008), gender (Lumpkins *et al.*, 2008), genotype (Zhao *et al.*, 2013), housing condition (Nordentoft *et al.*, 2011), floor litter (Torok *et al.*, 2009), feed restriction (Callaway *et al.*, 2009), stocking density (Guardia *et al.*, 2011) and age (Corrigan *et al.*, 2015). For example, *Firmicutes* are dominant in young chickens, while the *Bacteroidetes* are most common in adult birds (Callaway *et al.*, 2009). In layers, four different profiles of caecal microbiota have been identified from the day of hatching until 60 weeks of age (Videnska *et al.*, 2014).

Campylobacter is not simply a food contaminant but a member of the chicken gut microbiota and, as such, strategies targeting its reduction ought to consider ubiquitous microbiota modulation (Hermans *et al.*, 2011). Currently, many strategies have been tested, targeting microbiota modulation, such as probiotics, prebiotics and faecal transplantation (Roberfroid *et al.*, 2010; Fuentes and de Vos, 2016; de Cesare *et al.*, 2019) as alternative antimicrobial

strategies, which produce beneficial effects for the host whilst controlling zoonotic agents such as *Campylobacter*.

Antimicrobial resistance (AMR) amongst pathogens has been classified as a significant public health threat (Hofer, 2018). One of the main culprits for the increasing and persistent AMR spread is the misuse and overuse of antimicrobials (Dadgostar, 2019), for example, the administration of antimicrobials as growth promoters (AGPs), which is linked to increased performance and selection of resistant bacterial species (Cardinal *et al.*, 2020). Before AGPs were banned in most countries, they were commonly administered to birds; however, their mode of action towards increased performance had not been fully understood. It has been reported that some antimicrobials tend to enrich butyrate and lactate producers, probably explaining in part the effect on production (Robinson *et al.*, 2019). AMR distribution in livestock, including the poultry industry, not only represents a dangerous reservoir for the contamination and spread of AMR genes through the environment (Osman *et al.*, 2018) but it also represents a risk factor associated with the passage of resistant bacteria to humans (Bortolaia *et al.*, 2016). This is especially important in the context of zoonosis and horizontal gene transfer (Nhung *et al.*, 2017), likely to be allowing the incorporation of AMR genes from zoonotic pathogens into a member of the human gut microbiota (Sørensen *et al.*, 2001), and when considering *Campylobacter*'s possible resistance to fluoroquinolones, erythromycin, clindamycin, kanamycin and ampicillin (Luangtongkum *et al.*, 2006).

Several strategies have been adopted to reduce the spread of AMR in poultry production, including increased antimicrobial surveillance, decreased use of antimicrobials, better management strategies and finding alternatives to antimicrobials (Apata, 2009). The following sections describe the current most common antimicrobial alternatives used to lower *Campylobacter* concentration *in vivo* in poultry.

5.3 Antimicrobial Alternatives Against *Campylobacter*

5.3.1 Probiotics

Probiotics are defined as 'live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host' (FAO and WHO, 2001). This therapeutical approach is characterized by the administration of living microorganisms with a known beneficial effect on the host. Probiotics have been isolated from various sites, such as the gut and traditional fermented foods, and mostly belong to genera *Lactobacillus* and *Bifidobacterium*, although there are also some members of *Bacillus* and the yeast *Saccharomyces*, among others (Chaudhari and Dwivedi, 2022). Probiotics are thought to have several advantages, such as gut colonization whilst the probiotic is metabolically active, host health improvement either directly or indirectly by modulating other symbionts, relatively cheap production costs and safety given by the control over the strains used (Gaggìa *et al.*, 2010). Probiotics can be administered at different stages of the chicken's life following both temporal and longitudinal

microbiota dynamics (Stanley *et al.*, 2014). It can directly affect health and performance, for example, by influencing cellulose and fat digestibility (Rubio *et al.*, 2015). The main mechanisms of action of probiotics can be summarized in the modulation of the microbial communities and production of active substances, such as bacteriocins and organic molecules, the influence of the immune function and interaction with the intestinal mucosa, aggregation with pathogens and competition for nutrients (Tiwari *et al.*, 2012). Owing to these reasons, probiotics could modulate pathogens' establishment and spread and are therefore considered antimicrobial alternatives (Barba-Vidal *et al.*, 2019). Many studies have therefore focused their attention on the effect of different probiotic strains on *Campylobacter* (Saint-Cyr *et al.*, 2016; Deng *et al.*, 2020). However, probiotic beneficial effects are not only limited to lowering *Campylobacter* levels in the avian gut but are also extended to reducing environmental contamination at farms and ultimately contributing to enhanced carcass hygienic indices (Smialek *et al.*, 2018).

In a study from 2010, *Lactobacillus plantarum* PCS20 and *Bifidobacterium longum* PCB133 were independently tested in broilers, resulting in evidence showing that *B. longum*, but not *L. plantarum* was successfully found in excreta after ~21 days and was shown to significantly reduce *C. jejuni* (Santini *et al.*, 2010). Another study indicated, however, that several *Lactobacillus* species, such as *L. salivarius, L. johnsonii, L. reuteri, L. crispatus and L. gasseri*, were not only able to inhibit the growth of *C. jejuni* but also reduced the expression of *C. jejuni* virulence-related genes (apart from *L. reuteri*) and increased macrophage activity (Taha-Abdelaziz *et al.*, 2019). These findings probably confirm what is known as probiotic strain selectivity, in which probiotics are species/strain specific toward definite pathogen strains within the appropriate eukaryotic cell-type environment (Wine *et al.*, 2009). Interestingly, whole-genome-sequencing analysis of *L. salivarius* SMXD51 revealed the presence of several genes known to be related to gastrointestinal survival and adherence, for example, genes coding for acid tolerance and aggregation-promoting factor or adhesion capacity (Kergourlay *et al.*, 2012), corroborating the importance of competition mechanisms to combat *Campylobacter* adhesion.

5.3.2 Prebiotic

Prebiotics are defined as selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits upon host health (Gibson *et al.*, 2010). Some commonly used prebiotics are fructo-oligosaccharides (FOS), mannan-oligosaccharides (MOS), galacto-oligosaccharides (GOS), inulin, lactulose and xylo-oligosaccharides (XOS). In addition, prebiotics modulate the microbiota towards the proliferation of species possibly beneficial for the host, for example, digesting dietary molecules otherwise usually escaping host digestion (Zimmermann *et al.*, 2001). Several plant-derived substrates have been proposed and tested as a prebiotic, such as resistant starch or small undigested

oligosaccharides, which seemed to affect the caecal concentration of bacterial SCFAs (Pan *et al.*, 2009). As well as the induction of SCFA production, other mechanisms of action of prebiotics are the contribution to pH homeostasis, lipid metabolism control, immunomodulation via binding the G protein-coupled receptor of the gut-associated lymphoid tissue or via interaction between some SCFAs and cytokines production, controlling inflammation and improving nutritional absorption (Gowrishankar *et al.*, 2021). For these reasons, prebiotics have been studied widely in the context of limiting *Campylobacter* concentration within the chicken gastrointestinal tract, as already reviewed (Kim *et al.*, 2019). Several studies have reported that using prebiotics alone did not reduce *Campylobacter* concentration. Indeed, MOS-based prebiotic was linked to increased villi surface area and reduction of *Salmonella* levels but not *Campylobacter* in turkeys (Rahimi *et al.*, 2019). Similarly, GOS given to challenged birds did not reduce *Campylobacter* but was linked to increased performance (Flaujac Lafontaine *et al.*, 2020).

In contrast, *Saccharomyces*-derived prebiotic was associated with a decreased level of *Campylobacter* in the caeca, increased body weight, average daily gain and average daily feed intake (Froebel *et al.*, 2019), and 2 g/kg MOS reduced ileal and caecal *C. jejuni* concentration at day 24 and 42 in broilers, whilst also increasing *Lactobacillus* in two locations and time points (Rostami *et al.*, 2022). These findings might suggest that prebiotics are unlikely to exert a direct effect on *Campylobacter*; however, their likely indirect effect could be mediated by specific microbial communities and their related molecules. Following this rationale, prebiotics that can lead to an enrichment of those populations with the known detrimental outcome on *Campylobacter* could be successfully applied to reduce *Campylobacter* levels *in vivo* in poultry production to reduce the zoonotic risk pre-harvest.

5.3.3 Synbiotics

The synergic combinations of probiotics and prebiotics, in which the latter selectively provides advantages to the former, are defined as synbiotics (Pandey *et al.*, 2015). As already reviewed by Malik *et al.* (2019), there are many examples of the beneficial effects of synbiotics both on pathogen reduction and improved performance. For example, the combination of *B. longum* and GOS had been reported to decrease *C. jejuni* abundance in excreta samples (Baffoni *et al.*, 2012), whereas a synbiotic based on *B. longum* and XOS reportedly resulted in a reduction of *C. jejuni* in caeca of broilers after continuous administration (Baffoni *et al.*, 2017). Importantly, synbiotics have been successfully tested against other zoonotic agents; for example, FOS used with *L. reuteri*, *Enterococcus faecium*, *Bifidobacterium animalis* and *Pediococcus acidilactici* was able to exert a beneficial effect against *S. enterica* infection in caeca of layers, whilst also increasing body weight (Luoma *et al.*, 2017). This evidence shows that probiotic strains and molecules that improve functional microbiota modulation could perform better than administering a single component based on the synergy of action.

5.3.4 Bacteriophages

Bacteriophages are intracellular viruses specifically infecting bacteria, with two main typologies: virulent phages characterized by a lytic life cycle (i.e. formation and liberation of virions via cell-lysis) and temperate phages with a lysogenic life cycle, through which viral DNA is incorporated in the bacterial chromosome (Clavijo and Flórez, 2017). Bacteriophages can therefore be used as a therapeutical approach to target *Campylobacter*, leading to its reduction *in vivo* (Zhang *et al.*, 2022). Different strategies have been tested so far, including single phage therapy (Jeon *et al.*, 2012), phage cocktails (Molina *et al.*, 2021), a phage–antibiotic combination (Tagliaferri *et al.*, 2019) or phage-derived enzymes such as depolymerases (Pires *et al.*, 2016) and endolysins (Schmelcher and Loessner, 2021). Both single- and cocktail-phage approaches successfully induced up to $\log_{10}$ CFU/g *C. jejuni* concentration reduction between one and four weeks after treatment in broiler birds (Fischer *et al.*, 2013). Other authors corroborated these findings by describing a reduction in *C. jejuni* abundance in broilers after oral treatment with *Campylobacter*-specific- phage cocktail, importantly without further affecting microbiota species (Richards *et al.*, 2019), or showing 2 log reduction in *C. jejuni* and *C. coli* counts in excreta of birds artificially exposed to *Campylobacter* spp. (Carvalho *et al.*, 2010).

5.3.5 Avian antimicrobial peptides

Avian antimicrobial peptides (AAPs) are small molecules of ~50 amino acids, part of the innate immunity of poultry, with cathelicidins and β-defensins representing the main families; their main mechanism of action is exerted either by their hydrophobicity, leading to cell lysis via formation of transmembrane pores and ion channels, or by the recruitment and activation host-immune cells (Nguyen *et al.*, 2021). Chicken β-defensins (e.g. AvBD1–14) show tissue-specific expression levels, with high mRNA levels of AvBD4 to AvBD7 found in chicken bone marrow but not in leukocyte extracts, and they have known activity towards Gram-positive and Gram-negative bacteria, and fungi, such as *C. jejuni*, *C. perfringens*, *S. aureus*, *C. albicans* and *S. cerevisiae* (van Dijk *et al.*, 2008). Cathelicidins have a critical role in the innate avian immune system; they are mainly expressed by epithelial cells or bone marrow (Wang *et al.*, 2020), and are grouped into four classes, CATH-1, CATH-2, CATH-3 and CATH-B1, with the truncated peptides CATH-1(6–26), CATH-2(1–15) showing less toxicity (Nguyen *et al.*, 2021).

The immunological role of AAPs in response to *Campylobacter* has been demonstrated by several authors, indicating their potential therapeutic efficacy, for example, as a feed additive upon *in-vitro* production of a recombinant. During a challenge trial with different concentrations of *C. jejuni*, it was demonstrated that there was a differential expression level of some β-defensins, apparently dependent on the initial inoculum concentration; for example, duodenal AvBD10 was up-regulated in birds given 10^6 CFU but down-regulated

in the ileum of the 10^8 CFU group (Garcia *et al.*, 2018). Interestingly, it was demonstrated that *Campylobacter* challenge was significantly associated with reduced expression levels of some β-defensins (i.e. AvBD3, AvBD4, AvBD8, AvBD13 and AvBD14) and cathelicidins (i.e. CTHL2 and CTHL3), when compared to *Salmonella*-challenged birds, probably indicating a different host-immune response to both bacteria with a possible explanation for the high colonization level of *Campylobacter* (Meade *et al.*, 2009). The importance of AAPs in intestinal resistance to *Campylobacter* colonization was underlined in a study assessing the expression levels of genes in chicken genetic lines resistant or susceptible to *Campylobacter* colonization, emphasized by the caecal up-regulation of both β-defensins 10 and 12 in resistant birds (Li *et al.*, 2010).

5.3.6 Bacteriocins

Bacteria also produce antimicrobial peptides, called bacteriocins, produced by Gram-positive and Gram-negative bacteria to inhibit the growth of other microorganisms (Benítez-Chao *et al.*, 2021). In nature, bacteriocins have several roles, such as assisting colonization by inhibiting competing microbes in an already occupied microenvironment, functioning as signalling peptides targeting both bacteria (e.g. quorum sensing) and the host's immune cells (Mohan, 2015). For these reasons, like AAP, bacteriocins represent rather interesting antimicrobial alternative molecules, which could be synthesised *in vitro* and the administration of which could either hinder *Campylobacter* growth pre-harvest or modulate the rest of the microbial communities, leading to beneficial effects for the host.

As already reviewed by Svetoch and Stern (2010), therapeutic bacteriocins could reduce poultry *Campylobacter* levels in the caeca of chickens from $>10^8$ CFU/g to non-detectable or very low levels. In another study, bacteriocins produced by *Bacillus circulans* were able to reduce *Campylobacter* concentration to non-detectable from ~$1x10^6$ in the caeca of challenged chickens (Cole *et al.*, 2006). Corroborating these results, two different experiments demonstrated that bacteriocins derived from *L. salivarius* added in-feed reduced caecal *Campylobacter* concentration (Stern *et al.*, 2006), also showing that 250 mg of purified bacteriocins/kg of feed was able to reduce pathogen concentration otherwise not affected by the administration of the probiotic-producing bacteriocins only (Stern *et al.*, 2008).

Similar molecules to bacteriocins and AAPs are produced by other groups of organisms, such as plants (thionins), e.g. compounds in the leaf of *Ferula gummosa* are active towards *C. jejuni* (Kafi *et al.*, 2022), amphibians (magainins) or insects (melittin) (Karpinski and Szkaradkiewicz, 2013). However, this chapter does not describe their applications within the topic of reducing zoonosis.

5.3.7 Cell-free supernatant

There has been considerable evidence showing that the protective role of probiotics was not only dependent on viable bacteria (Harb *et al.*, 2013). Indeed,

in addition to the aforementioned bacteriocins, there is a plethora of active extracellular antimicrobial molecules of bacterial origin, such as hydrogen peroxide, reuterin, nisin and pediocin, that can act synergistically to lead to effective microbial inhibition. This suggests the use of cell-free supernatant (CFS, i.e. the ensemble of all the bacterial molecules found in the supernatant after centrifugation) as an antimicrobial alternative (Beristain-Bauza *et al.*, 2016). The technical steps to produce CFS are relatively easy and include the propagation of the probiotic strain(s) of interest, the separation of the cellular component by centrifugation followed by filter sterilization of the supernatant and, finally, the lyophilization of the CFS for downstream application (Kim *et al.*, 2021).

In one study, CFS from *Lactobacillus fermentum* created a clear inhibition zone in a disc diffusion test on *C. coli* and *C. jejuni*, whereas *L. thermotolerans* CFS was effective only on *C. coli* and *L. reuteri* CFS was only effective on one strain of *C. jejuni* (Bratz *et al.*, 2015). The same study also showed no activity towards *Campylobacter* spp. of CFS from *E. faecium* or 13 *Bifidobacterium* strains tested. These findings clearly indicate specificity between the CFS molecules from specific probiotics and pathogens, probably pointing towards target-recognition-like mechanisms. The ability to use this therapeutic approach by targeting specific species is interesting and is accompanied by great potential for applications in both the poultry industry and human medicine. Conversely, a similar study assessed the growth of *C. coli* via optical density measurement whilst growing in incubation with different CFS concentrations from similar probiotic strains and demonstrated an inhibition at a concentration from 1:1 to 10:1 of *E. faecium*, *B. animalis*, *P. acidilactici* and *L. reuteri* (Mortada *et al.*, 2020). The findings from these two experiments seem to be in contrast and indicate that further research is needed, especially to classify the CFS molecular composition in correlation to its effect on *Campylobacter*. Moreover, CFS as a feed additive would represent a novel product reducing zoonotic load pre-harvest, thus improving gut health and lowering antibiotic usage and AMR spread.

5.3.8 Non-organic alternatives

Within this category, we describe molecules used *in vivo,* as a biocide or as a broad-spectrum antimicrobial alternative, which could help reduce the *Campylobacter* concentration pre-harvest, reducing the potential campylobacteriosis risk. For example, in-feed ferric tyrosine (TYPLEX® Chelate) successfully inhibited *C. jejuni* biofilm formation and reduced both *C. jejuni* and *E. coli* colonization in broiler chickens at market age. (Khattak *et al.*, 2018). In another study, peracetic acid (PAA), commonly used for its biocide effects (Kitis, 2004), was tested in broilers. PAA was generated via the administration of two precursors, sodium percarbonate (SP) and tetraacetylethylenediamine (TAED, Aga2 Tech), within physiological pH values, and showed antimicrobial properties *in vivo*, without releasing harmful by-products (Galgano *et al.*, 2023). Furthermore, triclosan, benzalkonium chloride, cetylpyridinium chloride, chlorhexidine diacetate and trisodium phosphate are amongst the non-organic

molecules investigated for their action on *Campylobacter.* It has been shown, however, that *Campylobacter* could develop efflux pump-mediated resistance to these compounds (Mavri and Smole Možina, 2013). The mode of action of non-organic alternatives is intervention dependent; for example, the precursor-derived PAA was formed by hydrolysis of SP and TAED in the upper gut and, once active, interacted with strong oxidizing activity towards bacterial membranes, leading to bacterial inactivation (Galgano *et al.*, 2021).

5.4 *In-vitro* Investigation of the Gut Microbiota

Depending on the research question and the investigation setup, the different approaches to studying the interactions and composition of gut microbial communities are generally classified as culture-based methods, culture-independent methods and mathematical modelling.

5.4.1 Culture-based methods

Culture-based techniques have many applications, including new species identification, dynamics exploration within experimental environments (Lagier *et al.*, 2015) and bacterial quantification (Khattak *et al.*, 2022). However, they require a strict setup, *e*.g, temperature, pO_2 and medium composition, specific for the tested species, or similar to chyme composition if the study is aimed at assessing microbiota dynamics within a specific gut location (Bindelle *et al.*, 2007). Culture-based experiments can be either in batch (Sánchez *et al.*, 2006) or in continuous systems (Tanner *et al.*, 2014), with a growing level of complexity in the latter, which allow the *in vitro* simulation of parameters such as retention time and therefore allow temporal dynamics closer to physiological ones (Galgano *et al.*, 2018). In addition, complex bioreactor types have been designed and tested, allowing the simulation of other factors, such as the interaction between microbiota and host cells (Zhou *et al.*, 2018).

5.4.2 Culture-independent methods

The entire microbiota composition can be explored with metataxonomic methods (Marchesi and Ravel, 2015), which could be based on the sequencing of marker genes such as one of the 16S rRNA gene regions (Chakravorty *et al.*, 2007), or on the sequencing of the whole genome (Medvecky *et al.*, 2018), with increasing level of details in the output and increasing costs.

5.4.3 Mathematical modelling

The typical bacterial growth curve, from lag to decline phase, can be modelled *in silico* (i.e. computationally) using specific equations (Monod, 1949),

which could be used in the context of a complex system of ordinary differential equations, generating simulations and predictions of the microbiota dynamics through experimental conditions (Kettle *et al.*, 2018; Galgano *et al.*, 2020). Mathematical models of gut microbiota are reliable computational tools that can anticipate changes caused by microbiota modulation, such as the administration of probiotics or prebiotics (Esser *et al.*, 2015) and therefore represent a valuable instrument in the study of the interaction of different intervention types such as the ones described in this chapter.

5.5 *In-vivo* Testing Methods

Reliable *in-vivo* infection models are required to test the efficacy of, for example, novel products for the inhibition of caecal colonization by *Campylobacter* in broilers. Three different infection models, 'natural', 'seeded litter', and 'gavaging' methods, have been successfully and reliably used to colonize 21-day-old broilers by 28 days of age with *Campylobacter* spp. (Khattak *et al.*, 2018, 2021). The natural infection model involves using *Campylobacter*-positive litter from commercial farms/external trials, facilitating the faecal/oral challenge route (Khattak *et al.*, 2017). However, the limitation of this method is the timebound need for outsourcing litter and the risk of unequal pathogen distribution within a pen. The seeded litter challenge model was therefore developed and used to horizontally transfer *C. jejuni* in birds within an experimental pen via inoculating the litter with a mixture of *Campylobacter* species and excreta in a specific medium, mimicking infection dynamics in commercial settings (Sandilands *et al.*, 2018). However, this procedure does not allow precise *Campylobacter* quantification per bird. In contrast, the oral gavaging method allows the administration of a precise amount of *Campylobacter* inoculum directly into the crop of chickens (Arsi *et al.*, 2015). Nevertheless, this technique requires a high level of expertise of trained personnel, accompanied by increased costs due to the individual manipulations of all the birds in the flock.

5.6 Pro and Prebiotics: Marketing Trends and Future Forecasts

The market for probiotics and prebiotics has seen a rapid and constant increase throughout the years since the launch of the first products (e.g. Yakult® and non-digestible fibres) through the 20th century.

The global probiotic market is segmented by product type, that is, food and beverages, dietary supplements and animal feed 'biotics'. According to the global market forecasts, the probiotic market is expected to grow at a compound annual growth rate (CAGR) of 7.5% from 2022 to 2030, with increased market share by food and beverages compared to animal feed (MAP, 2021a, b). An annual market growth (CGAR) of 14.9% from 2022 to 2030 is expected for prebiotics in the food and feed industry, mostly due to rising awareness regarding the benefits of functional probiotic foods, increased demand for high-nutrition and low-calorie processed food products, the ban of

in-feed antibiotics in livestock, and the known probiotic-driven beneficial effects in animals.

Among all segments, the lowest global growth is recorded in the animal feed sector, probably because of the strict regulations, the need for further testing of novel feed additives and the lengthy approval processes.

5.7 Conclusion

The current evidence suggests that the use of probiotics, prebiotics and a combination thereof is a valid and much-needed approach to lower the sub-therapeutic need for antibiotics in animals and thus aids towards lowering antimicrobial resistance and, ultimately, enhances food safety. The concept of using 'biotics' to promote human health is old but determining how they affect foodborne pathogens is still in its infancy. Although the reliable and commercially relevant *in vivo* infection models for *Campylobacter* spp. challenge in broilers have been established, concerns regarding the optimal dosing and understanding the delicate interaction between *C. jejuni* and the chicken immune system remains a challenge for the scientists. Therefore, further targeted research is required to understand the full potential of these antimicrobial alternatives in the context of campylobacteriosis.

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