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Necrotic enteritis and antibiotic-free production of broiler chickens: Challenges in testing and using alternative products

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A R T I C L E I N F O

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A B S T R A C T

The global trend towards raising broiler chickens without the use of in-feed antibiotics (IFAs) means that there is an ongoing need to develop alternative treatments capable of delivering the benefits that IFAs previously provided. IFAs supported the productivity performance of chickens and played a key role in maintaining their health. Necrotic enteritis (NE) is an important disease of broilers that affects health, productivity, and welfare, and was previously well controlled by IFAs. However, with the reduction in IFA use, NE is resurgent in some countries. Vaccines and various feed additives, including pre-, pro-, and postbiotics, phytobiotics, fatty acids, and phage therapies have been introduced as alternative methods of NE control. While some of these feed additives have specific activity against the NE pathogen, *Clostridium perfringens*, most have the more general goal of reinforcing gut health. Extensive reviews of the effects of many of these feed additives on gut health have been published recently. Hence, rather than cover previously well reviewed areas of research this review focuses on the challenges and pitfalls in undertaking experimental assessment of alternative NE treatments and translating laboratory research to real world commercial production settings. The review is based on the author's particular experience, reading, thoughts, and analysis of the available information and inevitably presents a particular understanding that is likely to be at odds with others thinking on these issues. It is put forward to stimulate thinking and discussion on the issues covered.

1. Introduction

Necrotic enteritis (NE) is a significant burden on the poultry industry. The disease causes damage to the gut, resulting in diminished efficiency of nutrient use and hence reduced productivity. The production losses caused by NE and the currently applied methods of control have been estimated to cost the global broiler industry approximately six billion US dollars per annum (Wade and Keyburn, 2015). Clinical and sub-clinical forms of NE have been recognised (Van Immerseel et al., 2004). The clinical form results in acute disease that leads to the death of birds and is therefore obvious to poultry growers. The sub-clinical form of the disease is less obvious as it is not accompanied by a large spike in mortalities but rather leads to a reduction in feed conversion efficiency and is accompanied by dysbiosis of the gut microbiota (Antonissen et al., 2016; Lacey et al., 2018; Stanley et al., 2012; Yang et al., 2021). The productivity suppression triggered by the sub-clinical form of NE results in the biggest economic losses to the industry, caused by the disease. Poultry producers need to be vigilant in monitoring for the disease, use management approaches that reduce the chances of disease outbreaks, and rapidly respond when disease outbreaks are detected.

In previous decades, NE was generally well controlled by in-feed antibiotics (IFAs). However, with the growing problems associated with the increasing incidence of antibiotic resistant pathogens, particularly in human health settings, and the consumer demands for animal products produced without antibiotics, there has been a rapid response from the global poultry industry to move away from the routine use of IFAs. The shift away from IFAs has resulted in reduced productivity and increased incidence of NE in some field studies (Gaucher et al., 2015) but other studies have demonstrated

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that it is possible to maintain the health and productivity of flocks using IFA-free practices (Parent et al., 2020). With the strong industry moves away from IFAs, other approaches to deal with the ever-present threat of NE are needed. A wide range of feed additives and other types of treatments have been investigated and many products are now sold into this market to deal with NE.

2. *Clostridium perfringens* – the cause of necrotic enteritis

NE is caused by the Gram-positive bacterium, *Clostridium perfringens* (Bennetts, 1930; Parish, 1961). To be well placed to devise and test new IFA-free approaches for NE control, it helps to know as much as possible about NE pathogenesis, predisposing factors, and *C. perfringens* virulence. This knowledge enables informed decisions to be made about which strains are relevant to use in product assessment trials and how NE disease induction trials are best implemented. *C. perfringens* not only causes NE but is also the causative agent of a range of mainly gastrointestinal diseases of humans and animals. Each disease is caused by a subset of *C. perfringens* strains that is specific for a particular host and disease spectrum. As cyto-active toxins are the principal drivers of *C. perfringens* pathogenesis, the carriage of specific toxin genes is the hallmark of virulent strains for each disease and host (Rood et al., 2018; Uzal et al., 2014). In the case of *C. perfringens* strains that cause NE in chickens, the most important virulence factor is the necrotic enteritis toxin B-like (NetB) toxin (Keyburn et al., 2008). Other toxin encoding genes are also found in NE causing strains, but their roles, if any, in disease pathogenesis remain unclear (van Asten et al., 2009; Drigo et al., 2008; Lacey et al., 2019). Alpha-toxin is encoded by all *C. perfringens* strains and is an important virulence factor in some diseases caused by *C. perfringens*, for example gas gangrene, but an analysis of gene knockout mutants has shown that it is not an essential virulence factor in NE (Keyburn et al., 2006; Navarro et al., 2018). The toxin perfringens large (TpeL) toxin encoding gene is carried by some NE strains, and it has been suggested that it may increase the virulence of strains, but that still remains to be definitively proven (Coursodon et al., 2012; Gu et al., 2019).

The scientific literature regarding NE pathogenic strains of *C. perfringens* has been muddied by problems with the interpretation and reproducibility of studies. The most robust way to investigate the role of toxins in pathogenesis is to compare isogenic strains, with specific knockout mutants compared to the wild-type parent strains. This approach has only been used to demonstrate the roles of alpha-toxin and NetB toxin and the effects on virulence of a few other genes, *cnaA*, *zmpA*, and *zmpB*, but has not yet been used for TpeL – another toxin that has been suggested to increase the virulence of strains (Keyburn et al., 2006, 2008; Wade et al., 2016, 2020). Researchers have tried to draw conclusions regarding the importance of toxins from the toxinotypes of strains isolated from NE diseased birds and healthy birds, but this approach is fraught with difficulties as birds, both healthy and diseased, can carry multiple strains of *C. perfringens*. Hence, source of isolation is insufficient to be able to classify strains as virulent or non-virulent. The only way to definitively assess isolates is to put them back into birds in a validated NE induction model. The results from infection experiments are clear when the appropriate controls are included – only strains carrying the *netB* gene can reproducibly induce NE (Keyburn et al., 2010; Prescott et al., 2016; Smyth and Martin, 2010). Again, with experimental disease induction models of NE there are pitfalls for the inexperienced researcher. Two issues are important. Firstly, because *C. perfringens* is ubiquitous in the environment and is often found in the gut of health birds, to be certain of infection study results, *C. perfringens* must be reisolated from the NE lesions of infected birds and shown to be the same isolate used for infection. Otherwise, any disease that is noted could have resulted from an extraneous *C. perfringens* strain rather than the strain being tested. Secondly, when the *Eimeria* infection predisposition model of NE induction is used (and this is the most widely adopted model), it is important to differentiate between coccidiosis lesions and NE lesions. A tell-tale sign of mis-scoring of lesions can be seen in reports in which NE lesions are scored weeks after infection with *C. perfringens*. In experimental NE disease induction models, the NE lesion scores usually peak the day or two after infection and then rapidly resolve, with no NE lesions visible a week after *C. perfringens* infection. Lesion scores recorded more than a week after *C. perfringens* infection are likely to be coccidiosis lesions caused by the second round of replication of the predisposing *Eimeria* infection applied to the birds before the *C. perfringens* infection. It is of little value to test alternative NE treatments in NE models systems that are not accurately interpreted. Also, animal trials that use the *Eimeria* predisposition model and only use productivity scores to assess treatment performance may be misled by the performance suppression caused by overdosing with attenuated *Eimeria* strains, rather than resulting from NE. Such difficulties are important to consider when designing and interpreting experiments aimed at determining the efficacy of potential non-antibiotic treatments against NE.

The accumulating knowledge about the toxins and other factors that determine the virulence of *C. perfringens* strains has helped to refine the understanding of *C. perfringens* dynamics in healthy poultry and in NE diseased birds. As mentioned previously, *C. perfringens* is ubiquitous in the environment and is very commonly found in the guts of healthy animals, including chickens. It had been hypothesised that any change to gut health that resulted in an increase in *C. perfringens* colonisation levels was potentially sufficient to trigger the onset of NE (Drew et al., 2004). However, most of the *C. perfringens* commonly found in the gut of chickens do not carry key virulence factors and are best thought of as commensal organisms with very little if any pathogenic potential. For NE to arise, the *C. perfringens* population that expands on disruption of the gut must encode essential virulence factors such as NetB. The *netB* gene is carried on a conjugative plasmid and so it can be transferred into commensal *C. perfringens* strains and convert them into virulent strains, and it has been shown that this transfer process can occur in the gut of chickens (Lacey et al., 2017). Although infection and expansion of the population of a NetB-expressing virulent strain of *C. perfringens* is necessary, it is not sufficient to induce disease, other predisposing factors must also be present (Moore, 2016). The most significant predisposing factor is gut epithelial damage caused by *Eimeria* infection, but many other factors can contribute to the likelihood of NE developing. An understanding of predisposing nutritional, microbiological, immunological, biochemical, environmental, and management factors can inform the use of products and approaches that can be applied to reduce the probability of NE arising in a flock (Emami and Dalloul, 2021).

In the simplest terms, NE can arise when a pathogenic strain of *C. perfringens* invades, colonises the gut, and then increases in abundance and produces toxin(s) that damages the gut epithelial layer, thus compromising gut integrity and function. An effective way to prevent NE is to disrupt the colonisation and expansion of *C. perfringens* in the gut. Antibiotics could directly suppress *C. perfringens* populations and several classes of other NE treatments, for example bacteriophages and some probiotics, may have similar direct effects (Bae et al., 2021; Elwinger et al., 1992, 1998; Keerqin et al., 2022). Expansion of the *C. perfringens* population is known to be influenced by several nutritional and physical factors and so modulating these factors can reduce the chances of NE occurring. Intestinal damage, either caused by *Eimeria* infection and

resulting in cell lysis and serum leakage, or high protein rations can promote *C. perfringens* growth. Therefore, coccidiostats, coccidiosis vaccines, and diets without excessive protein content are important tools in controlling NE. High viscosity and long passage time of the chyme may also result in increased *C. perfringens* numbers in the gut, therefore nutritional approaches (e.g., use of grains that produce less viscous chyme) and use of additives, such as enzymes to breakdown complex carbohydrates, to reduce viscosity and passage time, can be used to address some of these issues. Many other non-IFA approaches to NE control have been studied and are sold into the broiler production market.

3. Alternatives to IFAs for NE control

There are three broad approaches to NE control without IFAs; management practices, feed additives, and vaccines (Fig. 1). Management practices encompass the housing design, environmental controls, stocking density, litter condition, nutrition/feed formulation and supply management, water quality and management, and biosecurity. Feed additives include a wide range of products, such as prebiotics, probiotics, postbiotics, and phytobiotics. Each of these categories include many different products with a range of different modes of action. The third category of IFA-independent control methods is via vaccination. Many experimental NE vaccines have been reported but currently only a live *Salmonella* vectored vaccine (AvertNE), delivering alpha-toxin and NetB antigens (Wang et al., 2022), is commercially available, but only in limited regions (http://www.huvepharma.us/product/avert-ne/). Around 2010, another NE vaccine, NETVAX, an oil in water emulsion toxoid used as a maternal vaccine in broiler breeders, was marketed in North America, but was soon discontinued because of low efficacy (Crouch et al., 2010; Gobbi, 2008). The low efficacy was unsurprising in retrospect as the vaccine was made from a bovine derived toxinotype A strain of *C. perfringens* that did not carry key virulence factors relevant to NE. This outcome demonstrates the importance of understanding *C. perfringens* virulence and NE pathogenesis when designing and testing alternative therapeutic or prophylactic products. NE vaccines are further discussed below. Vaccines against the major predisposing *Eimeria* infections are widely available from several commercial suppliers.

Each of these ways of reducing the impact of NE, and many of the wide range of product types available, have been recently reviewed in an extensive collection of publications (Abd El-Hack et al., 2022; Alizadeh et al., 2021; Ayalew et al., 2022; Caly et al., 2015; Gadde et al., 2017; Kalia et al., 2022; Mehdi et al., 2018; M'Sadeq et al., 2015; Rahman et al., 2022; Williams, 2005; Zhu et al., 2021). Rather than to repeat already well reviewed areas of research, this review will briefly mention those approaches that have been the subject of recent up-to-date reviews, go into more detail for potential treatments not yet well reviewed, and then cover some of the difficulties and pitfalls that may be encountered

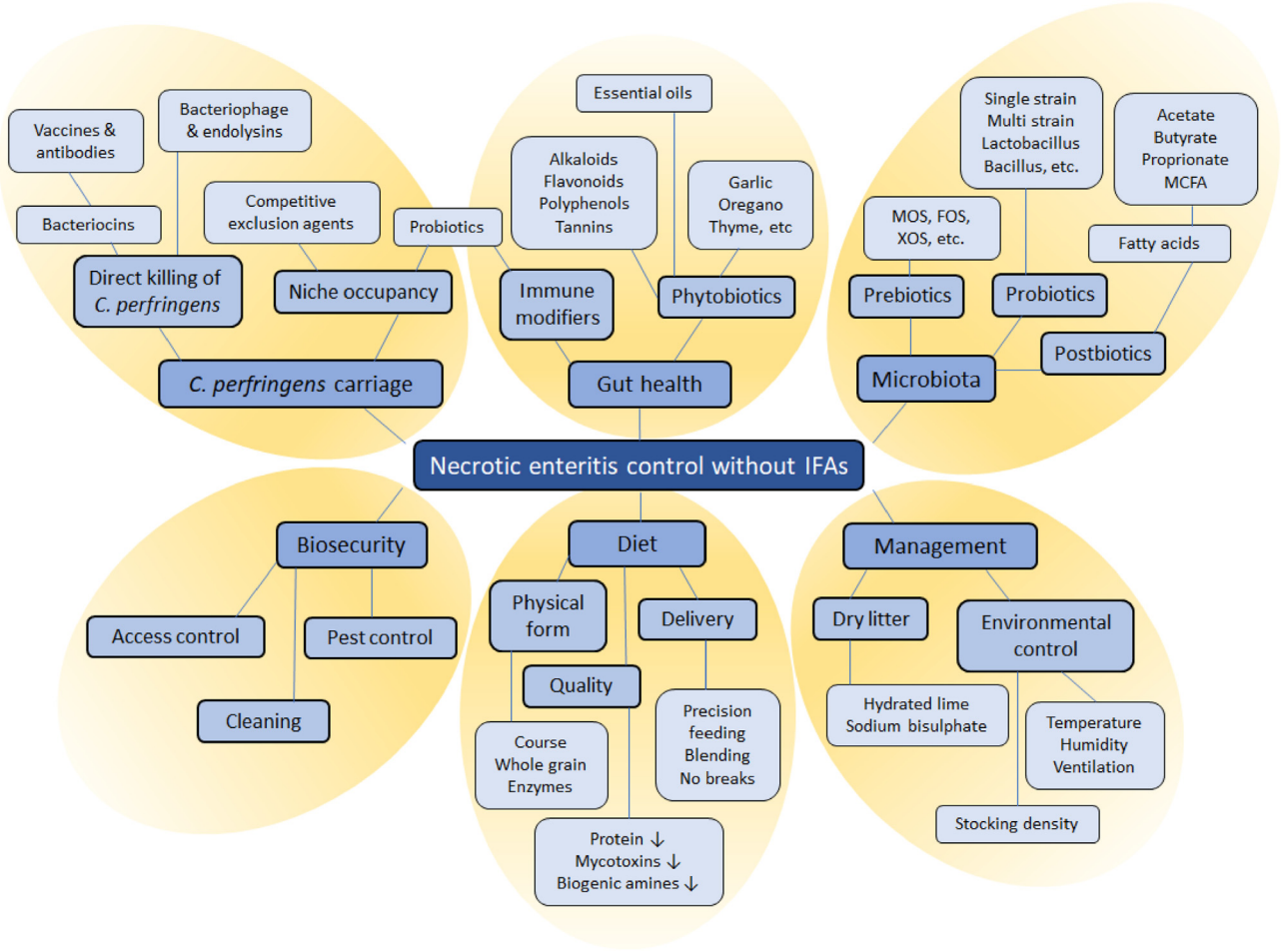

Fig. 1. Necrotic enteritis (NE) control without in-feed antibiotics (IFA). Some of the major products and management approaches that can be used to control NE. FOS = fructo-oligosaccharides; MOS = mannan-oligosaccharides; XOS = xylo-oligosaccharides; MCFA = medium chain fatty acids.

in the research, development, and testing of products, and how the results might translate to commercial use.

4. Management practices

Good management practices and animal husbandry are the starting points to control the threat of NE (Tsiouris, 2016). Healthy birds, good environmental conditions, and biosecurity ensure that birds are as robust as possible and less susceptible to NE than birds raised in sub-optimal conditions. Sub-optimal conditions can result in immunological stress, disrupted gut microbiota, colonisation and over-growth of *C. perfringens*, and sub-clinical or clinical NE. Feed quality and supply management are particularly important. It has long been known that feed composition and physical form can have a profound effect on the susceptibility of broilers to NE (Branton et al., 1987; Riddell and Kong, 1992). There are multiple mechanisms by which feed quality and management can predispose or protect from NE. The physical properties of the feed (e.g., particle size) and the resulting chyme (viscosity, pH) can change the passage time through the gut, and the oxygen levels, and produce conditions that are favourable for *C. perfringens* colonisation and multiplication (Moran, 2014). Grains such as wheat, barley, and rye can make broilers more susceptible to NE, but in certain regions these grains are an essential major component in diets because of price and availability considerations and so their use cannot be avoided. The addition of enzymes to break down complex carbohydrates can reduce the potential problems caused by grains that cause high viscosity and longer passage times (Kim et al., 2022). Similarly, proteases can improve the digestibility of protein in the diet (Park et al., 2020). Feed formulations can have a large effect on the composition and complexity of the gut microbiota (Crisol-Martínez et al., 2017), which in turn can alter susceptibility to NE (Antonissen et al., 2016). Feed composition, and in particular contaminants such as mycotoxins and biogenic amines, can directly damage the gut and provide an entry point for *C. perfringens* attachment and induction of further damage to the gut (Antonissen et al., 2014; Barnes et al., 2001). Further research needs to be conducted, but it is likely that some recent trends in nutrition management of broilers, such as low protein diets and precision nutrition, may help in the control of NE (Hilliar et al., 2020; Kogut, 2022; Lee and Rochell, 2022; Moss et al., 2021). Temporary feed restriction has been reported to partially protect chickens from developing NE (Tsiouris et al., 2014), a surprising result given that overnight feed withdrawal is an important predisposing factor used in some experimental NE induction models (Keyburn et al., 2006) and it is known that fasting can compromise gut integrity (Gilani et al., 2021). Further research needs to be undertaken to resolve these seemingly conflicting findings. Other aspects of management, such as temperature, humidity, litter condition, and stocking density can influence broiler predisposition to NE (Tsiouris et al., 2015a, 2015b, 2018). Finally, there is evidence that there are host genetic influences of susceptibility to NE, Therefore, there is some hope that it may be possible to increase the resilience of birds to NE by genetic selection (Oh and Lillehoj, 2016; Swaggerty et al., 2016; Zahoor et al., 2018). However, at this stage, it is unclear whether genetic selection for traits such as the level of pro-inflammatory mediators, which influence NE susceptibility, will have positive or negative effects on other aspects of the birds' biology, productivity, and response to other pathogen challenges and vaccination.

5. Feed additives used to control necrotic enteritis

There is a plethora of feed additives that have been studied to understand effects on NE susceptibility or protection. Most of the feed additives that are currently in commercial use have broad effects that are not targeted specifically at *C. perfringens* but rather have more general influence on gut health and integrity, gut microbiota, and/or immune competence of the birds. Many additives have overlapping and interconnected effects and so the health or NE ameliorating effects can be generated in different ways and with diverse products (Granstad et al., 2020). For example, short chain fatty acids, in particular butyrate, can have positive effects by both supplying a preferred energy source for enterocytes and encouraging the development of a beneficial gut microbiota (Liu et al., 2021). Butyrate can be supplied directly to the gut as a chemical in feed, most effectively when supplied in a “protected” form that allows passage to the small intestine. However, other feed additives can indirectly increase butyrate levels in the gut, for example prebiotics supply the substrate from which butyrate can be produced by butyrate-producing bacteria that increase in abundance because of the prebiotics (Van Immerseel et al., 2017). Alternatively, butyrate-producing bacteria can be used as probiotics to directly supplement the gut microbiota (Onrust et al., 2015). Some phytobiotics can also encourage the growth of butyrate-producing bacteria, and finally some postbiotic preparations may contain butyrate or provide substrates from which butyrate can be produced (Aljumaah et al., 2020; Onrust et al., 2015). These interconnected effects also mean that combinations of products are also often seen as beneficial, for example, short and medium chain fatty acid products may be paired with some phytobiotics to support bird health and resilience to *C. perfringens* infections.

Just as some biological outcomes can be generated in different ways by a variety of different classes of product, some classes of product can function in a wide variety of ways. The probiotic category encompasses a diversity of products with widely varying compositions and modes of action (Moore, 2017). Many different species of bacteria and yeasts have been used as probiotics and products can contain single or multiple strains. Some microbial products are more complex and not fully defined but rather consist of cultured caecal microbiota from healthy birds. Most probiotics probably have multiple effects, rather than just targeting a single function. Some have direct antimicrobial activity against *C. perfringens*; some may act as competitive exclusion agents to reduce *C. perfringens* colonisation niches within the gut; others primarily function by improving gut microbiota composition and/or complexity, or by improving gut integrity, or improving immunological functioning of the gut. Thus, the probiotic category of products can have broad and varied positive effects on poultry health and resilience to *C. perfringens* infection.

Many of the feed additives have been reviewed in recent years. The recent literature includes reviews of the use of prebiotics for general health support (Adebowale et al., 2019; Jha et al., 2019; Regassa and Nyachoti, 2018), probiotics for both general health support (Alagawany et al., 2018; Lone et al., 2022; Rajput et al., 2020) and specifically as alternative products to ameliorate NE (Khalique et al., 2020; Kulkarni et al., 2022; Rajput et al., 2020), fatty acids for NE control (Gomez-Osorio et al., 2021), and phytobiotics for general (Abdelli et al., 2021; Biagini et al., 2022; Yang et al., 2009, 2015) and NE specific applications (Diaz Carrasco et al., 2016). Many of the products in these major categories of in-feed additives for NE control are commercially available. A few of the more novel and speculative in-feed additives are discussed below.

There are a few other alternative treatments that can be delivered in the feed that are more specifically targeted at *C. perfringens*. Bacteriophage (phage) are viruses that infect and kill bacteria. They are interesting therapeutic options because each phage has a very restricted host-range, usually only infecting a specific species of bacteria. This high specificity is an attractive property as it holds out the hope that they could be used to specifically kill *C. perfringens* without having any effect on other bacteria within the gut

microbiota, thus surgically removing the pathogen of concern without causing any unwanted disruption to beneficial bacteria. The potential use of phage therapies has been reviewed in general (Gildea et al., 2022; Joerger, 2003; Mills et al., 2017) and specifically for anti-clostridial activity (Venhorst et al., 2022). A number of studies have identified phage specific for NE derived strains of *C. perfringens* (Keerqin et al., 2022; Miller et al., 2010) and (Miller et al. 2010) were able to demonstrate a reduction in NE severity using phage therapy. Although an interesting alternative to IFA use, phage therapy does have some barriers to adoption. The high host specificity and the common occurrence of phage resistant bacterial mutants means that any viable phage therapy product would need to contain a collection of multiple phages with a variety of different receptors. A refinement of the phage therapy concept is the use of the specific enzymes that phages use to lyse the bacterial host. These endolysin enzymes have targeted antibacterial activity (Carvalho et al., 2017) which may, in the future, be able to be adapted to help with NE control.

Just as endolysins are derived from bacteriophages, there is another class of antimicrobial protein, bacteriocins, produced by bacteria, that could potentially be adapted to treat NE. One of their functions is to act as "biological warfare" agents that help a producing bacterium to fight against competing bacteria for occupancy of an environmental niche. It is the production of bacteriocins that gives some bacterial strains the antimicrobial properties that make them attractive candidates as probiotics. There is extensive research into the use of bacteriocins as alternatives to conventional antibiotics, particularly with a view to using them to combat antibiotic resistant pathogens (Cavera et al., 2015), and potential use for food preservation (Johnson et al., 2018), but there has been relatively little investigation of their potential use in production animals or specifically for use as a treatment for NE (Ben Lagha et al., 2017).

Passive immunisation is another novel in-feed additive approach that has shown some promise for the amelioration of NE. This is an interesting approach as it avoids the timing difficulties in directly immunizing chicks. Rather than aiming to produce an immune response in chicks, egg yolk antibodies have been used to supply a readymade "immune response". Early work demonstrated that anti-*C. perfringens* antibodies harvested from the eggs of vaccinated hens could reduce *C. perfringens* numbers in the gut, but exacerbated intestinal NE lesion scores (Wilkie et al., 2006). Recent work has produced more encouraging results with indications of efficacy in reducing pathological symptoms (Abadeen et al., 2022; Khalf et al., 2016). It is not clear that this concept has been adequately tested as it is possible that more effective antibodies could be produced using alternative strains of *C. perfringens* and/or specific virulence-related proteins for vaccination. A further extension of this idea of providing immune molecules is the use of single chain antibodies. Single chain antibodies can be engineered from chicken antibodies and antibodies from other species, and they are naturally produced by some animals, including llamas and sharks (Könning et al., 2017; Sapats et al., 2003). The cost of producing and delivering purified antibodies is probably too high for routine use in poultry production and so live bacterial delivery has been investigated, with some success (Gangaiah et al., 2022). Gangaiah et al. (2022) delivered llama antibodies directed against the *C. perfringens* NetB toxin in a lactic acid bacterial strain, *Limosilactobacillus reuteri*, and showed a significant reduction in NE in an experimental disease challenge model.

6. Vaccination

Vaccination is the mostly widely used method for infectious disease prophylaxis in both humans and animals. Many bacterial and viral diseases are effectively controlled by vaccination (Kayser and Ramzan, 2021; Pastoret and Jones, 2004). Protection from many clostridial diseases of animals has been achieved using simple bacterin or toxoid vaccines (Abdolmohammadi Khiav and Zahmatkesh, 2021; Zaragoza et al., 2019). This simple approach to vaccine design has not been successful for NE, indicating that vaccination with the complex antigen mix produced during in vitro bacterial growth is insufficient to raise a protective immune response. Interestingly, the addition of recombinantly produced protein of the key virulence factor, NetB, to a bacterin vaccine resulted in approximately 80% protection from an experimental disease challenge (Keyburn et al., 2013b). This indicates that in vitro grown *C. perfringens* lacks sufficient levels of key virulence proteins that are needed to induce immune responses that can disrupt the pathogenic processes involved in NE development.

With simple conventional bacterin and toxoid vaccines not providing sufficient protection, it became obvious that other approaches to vaccine design were needed if a vaccine solution for NE management was to be developed. By understanding more about NE disease pathogenesis, key functions in the pathogenesis process could be targeted for vaccination. Before the major virulence factor, NetB, was discovered (Keyburn et al., 2008), it had been assumed that alpha-toxin, which is produced by all *C. perfringens* strains, whether capable of causing NE or not, was an important virulence factor for NE (Al-Sheikhly and Truscott, 1977). Hence, early vaccination efforts had focused on alpha-toxin as either part of complex bacterin vaccines or single subunit vaccines, but the experimental vaccines only delivered modest levels of protection (Cooper et al., 2009; Lanckriet et al., 2010). The finding that alpha-toxin can induce some level of protection, is not, in itself, evidence that it has any significant role in pathogenesis. It has been shown that, although alpha-toxin is generally regarded of as a secreted toxin, the protein is present on the surface of *C. perfringens* cells (Zekarias et al., 2008), and therefore provides a target for antibody binding and subsequent bacterial opsonization. With the demonstration that alpha-toxin is not an essential virulence factor in NE (Keyburn et al., 2006), there was a shift in research emphasis towards the identification of other *C. perfringens* encoded factors that may be important in disease pathogenesis and hence could be useful targets for vaccine development. The discovery that NetB was a major virulence factor in NE was followed by the reporting of various vaccine trials that used NetB as an antigen (Fernandes da Costa et al., 2013; Jang et al., 2012; Keyburn et al., 2013a, 2013b; Shamshirgaran et al., 2022). One NetB based vaccine, that uses an intricately engineered *Salmonella* strain to deliver both NetB and a truncated alpha-toxin (Wang et al., 2022) is now sold commercially in North America (https://www.huvepharma.us/product/avert-ne/).

Other antigens, besides toxins, have shown some protective efficacy when used as subunit vaccines, but generally with only low or moderate levels of protection. An alternative vaccination approach that targeted glycoside hydrolases has shown some promise (Duff et al., 2019), as has the use of sporulation proteins as vaccine antigens (Fu et al., 2022). Gene knockout experiments have demonstrated that, in addition to NetB, other proteins, including adhesin (CnaA) and zinc metallopeptidases (ZmpA and ZmpB) have a profound influence on the virulence of NE causing strains of *C. perfringens* (Keyburn et al., 2008; Wade et al., 2016, 2020). This information has been used to develop multi-valent recombinant vaccines that have shown promise in experimental vaccination trials (Katalani et al., 2020). It appears likely that the most efficacious NE vaccines will include multiple antigens that target different stages in disease pathogenesis (Mot et al., 2014; Wilde et al., 2019; Yuan et al., 2022).

As NE typically occurs in young birds, at around three weeks post-hatch, there is little opportunity to deliver NE vaccines by the

traditional injection route to provide protection in this period of greatest vulnerability. Conventional adjuvanted injectable vaccination of chicks has often been used in research aimed at identifying protective antigens but such a delivery method is not commercially viable in most countries and so effective mass application methods are needed. Alternative vaccination approaches that are likely to be the most suitable methods of delivery of NE vaccines for use in commercial broiler flocks include the use of live delivery vectors with spray or in-feed/water application at the hatchery, in ovo vaccination, and maternal vaccination of breeder stock. Conventional injectable NE vaccines could be used for maternal vaccination of broiler breeder hens, as there is evidence that maternal antibodies can protect growing broilers around the critical three-week period when they are most vulnerable to NE (Keyburn et al., 2013a).

A further vaccination approach that may provide a level of protection against NE is to vaccinate against the principal predisposing factor, *Eimeria* infection (Bangoura et al., 2014; van Eerden et al., 2022). An experimental bivalent vaccine that included antigens from both *Eimeria* and *C. perfringens*, has recently been reported to induce significant levels of protection against NE (Fatemi Motlagh and Mousavi Gargari, 2022). Vaccine strategies that target both *C. perfringens* and *Eimeria* are likely to be the ideal solution to reduce the impact of NE.

7. Testing of products that may ameliorate necrotic enteritis

There are many ways in which NE could be ameliorated or avoided without using IFAs and this is exemplified in the array of alternative products to IFAs that have been reported in the scientific literature and/or are available commercially (Adhikari et al., 2020; Caly et al., 2015). Products may have one or more of several modes of action:

- Direct targeted activity against *C. perfringens* or predisposing *Eimeria*,
- Influence microbiota composition to competitively exclude *C. perfringens*,
- Encourage beneficial gut microbiota development, and
- Improve general gut health and immunity.

Each of these different modes of action need to be tested and assessed in different ways and so there are many analytical techniques that have been applied (Fig. 2). Some of the modes of action can be initially assessed in vitro, for example direct killing of *C. perfringens* by bacteriocin expressing probiotics or bacteriophage therapies, but in the end all alternative treatment methods and products need to be evaluated in NE affected birds. Usually, efficacy is first investigated in an experimental disease induction model, but in some circumstances may be done directly in a field setting.

8. Experimental induction of necrotic enteritis

To undertake meaningful structured evaluations of non-IFA methods of NE control it is important to initially screen products and approaches in reliable and relevant test systems. At its simplest, this could involve application to normal healthy birds to ensure that no adverse effects are produced and monitoring of some key parameters, such as gut microbiota structure and metabolite (e.g., butyrate) levels, to determine if they are modified in a way that may be useful for NE control. Ultimately, the most convincing evidence for the worth of a product or management approach is derived from testing in a fully controlled experimental model that induces NE (Fig. 2). Experimental reproduction of NE is not easy (Cooper et al., 2010; Prescott et al., 2016). Simple infection with the causative bacteria is insufficient to reliably induce disease and so predisposing factors must be applied. The most widely used method to induce NE uses pre-infection with *Eimeria* as the predisposing factor (Al-Sheikhly and Al-Saieg, 1980; Bortoluzzi et al., 2019). An alternative model system in which fish meal has been used to produce high protein wheat-based feed as the predisposing factor has also been developed (Cooper et al., 2010; Drew et al.,

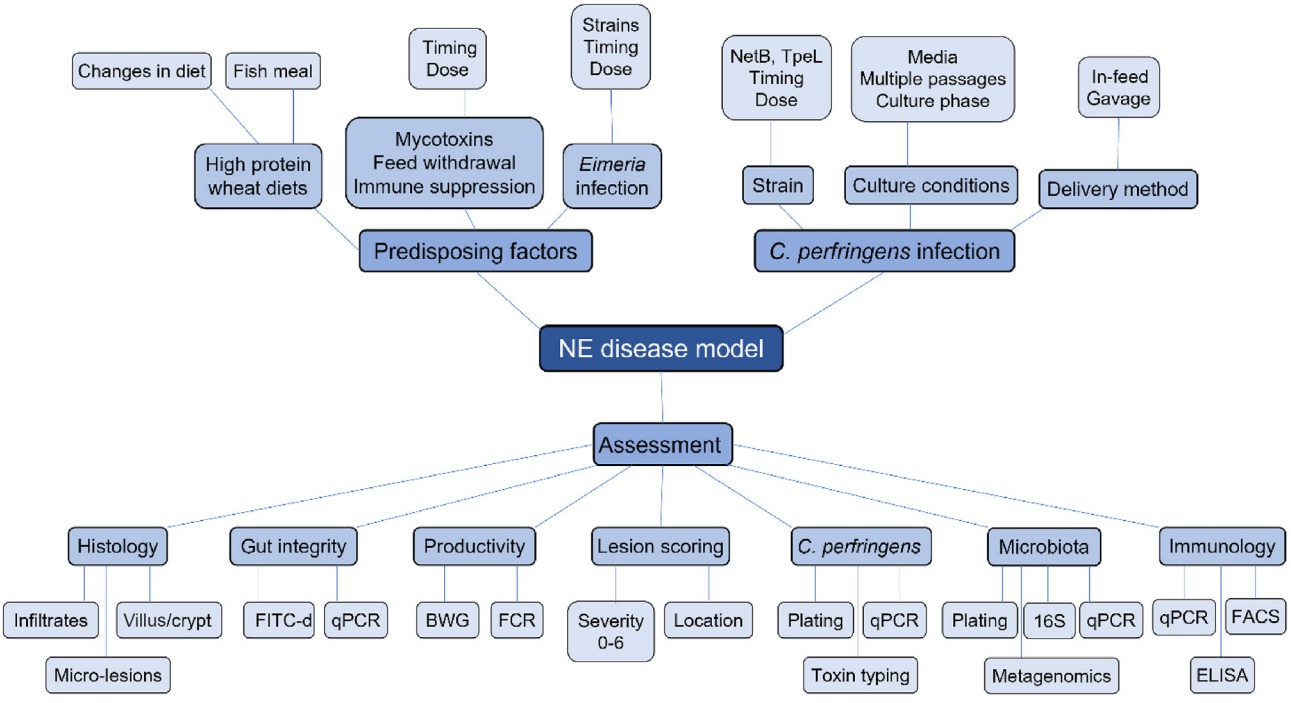

Fig. 2. Methods used to experimentally reproduce necrotic enteritis (NE) and analytical methods used to assess its impact. TpeL = toxin perfringens large; NetB = necrotic enteritis toxin B-like; FITC-d = fluorescein isothiocyanate-dextran; BWG = body weight gain; FCR = feed conversion ratio; FACS = fluorescence active cell sorting.

2004; Keyburn et al., 2006). These two model systems have been used to test a range of experimental vaccines and a wide range of feed additives. Each NE induction model has advantages and disadvantages which means that each model is more suited to testing particular types of products or management approaches to NE control. The fish meal model may not be a good choice for the testing of feed additives as the model uses such an extreme, atypical diet unlike any feed that would be used in normal commercial poultry production. The fish meal model has been mainly used to test the virulence of *C. perfringens* strains and mutants, and the efficacy of vaccines. The *Eimeria* predisposition model is the more relevant model for testing of IFAs as normal commercially relevant feed formulations can be used, but the model can result in high but somewhat unpredictable levels of mortality (not good for animal ethics consideration) (Ashall and Millar, 2014; Nunamaker et al., 2021), some immune suppression (not good for vaccine assessment) (Akhtar et al., 2015; Walston et al., 2016), and complications in distinguishing between effects of coccidiosis and NE (Williams, 2005). Both predisposing factors cause significant disruptions to the gut microbiota (Stanley et al., 2014; Wu et al., 2014). In some published work, it is likely that *Eimeria* induced gut lesions have been inadvertently reported as NE lesions. Lesion scoring can be carried out reliably but there is room for misinterpretation with the *Eimeria* predisposition models. There is further scope to refine the NE challenge models by judicious choices of *C. perfringens* and *Eimeria* strains, and the addition of other predisposing factors (Justino et al., 2022; Liu et al., 2020; Shanmugasundaram et al., 2022).

The NE induction methods have evolved over time and in experienced hands can deliver consistent and reproducible results. However, for experimental purposes, in particular to provide adequate statistical power for experiments, the challenge processes are designed to induce disease in most of the birds that are challenged. This is different to the typical disease outbreak situation where only a proportion of birds show overt disease symptoms. Another large variable in different iterations of the NE induction methods, is the *C. perfringens* strains and dosages that are applied, with some models using bolus gavage doses given repeatedly over several days, while others have used more gradually delivered, but larger doses, via the feed. None of these methods accurately reproduce the natural trickle infection process that is likely to occur in the field, and the timing of exposure may also differ from what would be expected to happen in field conditions. Similarly, the extreme application of predisposing factors used in many NE models, such as large bolus doses of *Eimeria* or extreme levels of protein do not replicate the typical circumstances that occur in well managed commercial flocks. It is not clear how the experimentally applied NE induction models could be modified to better replicate field infections, but it emphasises that in the end the in-field testing under natural infection and predisposition settings is the final indicator of the value of any applied product or control measure.

9. Monitoring for unintended consequences of alternative treatments

Many of the products that are offered as alternatives to IFAs to reduce the impact of NE modify the composition and/or metabolism of the gut microbiota. In developing and applying control measures for *C. perfringens*, one consideration that is often overlooked is that many strains of taxonomically related bacteria are commensal organisms within the chicken gastrointestinal tract and may play significant roles in promoting the heath of chickens by producing useful metabolites, such as short chain fatty acids, positively interacting with the chicken immune system, and acting as agents that occupy ecological niches and exclude colonisation by pathogenic strains (Guo et al., 2020; Stanley et al., 2013a; Svejstil et al., 2019). Caution needs to be applied when considering the use of control measures that may have wider impacts than just on pathogenic *C. perfringens* strains. An example of the potential pitfalls that can be encountered when developing alternative treatments for NE can be found in many in vitro analyses of bacteria that have been tested for use as probiotics to address NE. The direct killing activity against *C. perfringens* is frequently assessed but generally no further analyses are undertaken to determine the probiotic's effects on beneficial groups of bacteria. *Lactobacillus* strains are often developed as probiotics yet the bacteriocin driven antimicrobial activity of such strains, although extending to *C. perfringens*, are most commonly directed at other *Lactobacillus* species, bacteria which are generally regarded as beneficial (Eijsink et al., 2002; Todorov et al., 2020). Such interactions with potentially useful commensal bacteria are rarely assessed. In the end, in vivo assessment in birds does partially address this issue, but it may be possible to select useful strains more efficiently, at an earlier stage in screening and development, if a wider view is taken of the antimicrobial activity spectrum of products.

10. Conclusions and perspectives

Many studies, using a wide variety of feed additives (pre-, pro-, postbiotics, fatty acids, phytogenics, etc.), have demonstrated some level of efficacy in reducing the incidence of NE in either experimental infection models or, more rarely, in field trials. Despite the wide range of somewhat effective treatments that are available, NE still remains a problem in many countries. None of the current alternatives appear to be as effective, cheap, reliable, and as easy to apply as IFAs. But we cannot return to the use of antibiotics because of the perceived heightening of risk to human health management that would entail. Good experimental outcomes in controlled NE induction trials do not always translate into reliable performance in the field. The reasons for this are likely to be many and varied. They range from intrinsic variation in the products used, for example some phytogenic products can vary widely in the effective concentration of active ingredients (Cross et al., 2007), to the ongoing challenge of requiring effective action in a variable gut environment in which the products must function (Stanley et al., 2013b). As many products function by modifying the composition and/or metabolic properties of the microbiota, the variability in the underlying microbiota of birds is likely to have a significant impact on whether a treatment is successful in a particular flock. The gut environment of broilers is influenced by many factors including, the resident microbiota, metabolites, different feed ingredients, water quality, and environmental factors. Therefore, commercial flocks may have different gut environments to those encountered in experimental flocks in which products were originally tested. Hence, there is a need for products to be tested in different production environments to determine their overall effectiveness. It is likely that some products will be suitable for general application under diverse conditions whereas other products may be more tailored for specific production environments and particular microbiota compositions. It is possible to evaluate product performance in a limited range of production scenarios in experimental settings, e.g., wheat- versus corn-based diets, different genetic lines of birds, different housing, etc., but the ultimate test of products is out in the diverse environments encountered in the field in commercial settings. The difficulty for commercial poultry growers is that such field results, particularly if negative, are not widely reported and so they often must rely on anecdotal reports from others in the industry or on their own experience, which can be expensive and time consuming to establish.

Alternatives to antibiotics that do not rely on the complex and often variable conditions and interactions within the gut microbiome may be more widely reliable products with more consistent and predictable performance. Therefore, vaccines that prime the chicken immune system to specifically combat NE may be the most effective solution if high efficacy vaccines can be devised and built, based on the increasing knowledge about *C. perfringens* virulence, NE pathogenesis, and vaccine design principals. This is an ongoing challenge for the research community and vaccine manufacturers. The currently available management approaches and many of the feed additive products promoted for NE control have wider application for the establishment and maintenance of general flock health and productivity and so have important applications in the poultry industry beyond just control of NE.

Author contributions

Robert Moore: Conceptualization, Literature curation, Writing, Reviewing, Editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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