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Preventing Necrotic Enteritis in Broiler Chicken

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Abstract: The use of antibiotics as growth promoters in animal feed, numerous studies have been published describing alternative strategies to prevent diseases in animals. A particular focus has been on prevention of necrotic enteritis in poultry which caused by *Clostridium perfringens* (*C. perfringens)* by the use of microbes or microbe-derived products. Microbes produce a plethora of molecules with antimicrobial properties and they can also have beneficial effects through interactions with their host. Here we review recent developments in novel preventive treatments against *C. perfringens*-induced necrotic enteritis in broiler chickens that employ yeasts, bacteria and bacteriophages or secondary metabolites and other microbial products in disease control.
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1. Introduction

Necrotic enteritis and broiler chickens

Clostridium perfringens (C. perfringens)-induced necrotic enteritis (NE) in chickens leads to sudden death, with mortality rates up to 50% **(Kaldhusdal and Løvland, 2000; McDevitt et al., 2006; Lee et al., 2011b).** More importantly, *C. perfringens* is also responsible for subclinical infections, associated with chronic damage of the intestinal mucosa. Such subclinical infections cause problems such as lower performance and reduced weight gain, which have dramatic economic consequences **(Elwinger et al., 1992; Kaldhusdal et al., 2001; Skinner et al., 2010).** The cost of NE worldwide was estimated to 2 billion dollars per year, which includes not only direct loss due to broilers deaths, but also veterinary and cleaning costs **(Van der Sluis, 2000; Timbermont et al., 2011).**

Clostridium perfringens is almost always found in healthy chickens, although at levels less than 10^5 cfu/g intestinal content. The ability of the bacterium to cause disease is linked to several predisposing factors that affect intestinal conditions and create a favorable environment for proliferation. Perhaps the most important of these factors is the incidence of coccidiosis **(Al-Sheikhly and Al-Saieg, 1980; Craven et al., 2001; Williams, 2005; Si et al., 2007).** NE incidence and the mortality rates are higher when chickens are co-infected with *Eimeria*, a causal agent of coccidiosis **(Shane et al., 1985; Baba et al., 1992).** The feeding diet has been shown to be another factor favoring disease, through an influence on the properties of the intestinal content such as viscosity and the presence of non-digestible polysaccharides, the GI tract transit time and the intestinal pH (**Annett et al., 2002**; **Drew et al., 2004; Moran, 2014**). For example, diets rich in wheat or fish proteins are known to increase the risk of necrotic enteritis **(Annett et al., 2002; Drew et al., 2004).**

The rapid death (within 24 h) of chickens with NE often prevents the treatment of the disease. Antibiotics have been commonly used worldwide as growth promoters and for prophylactic treatment of *C. perfringens*-induced NE in poultry. However, with the European ban on antibiotics (feed additives regulation 1831/2003/EC), which took effect in January 2006, alternatives to antibiotics became essential in order to prevent NE occurrence and the consequent economic losses for the poultry industry. Preventive treatments can take the form of actions on predisposing factors, such as coccidiosis prevention, diet modifications, or improving overall cleanliness and hygiene. Alternatively they can directly target the causal agent of the disease by controlling the proliferation, colonization and persistence of virulent strains of *C. perfringens* or interfering with virulence and pathogenicity factors. *C. perfringens* infections can be reduced or abolished by using natural feed additives, such as probiotics (yeasts or bacteria), plants (**Engberg et al., 2012**), molecules of plant origin [for example, essential oils (**Mitsch et al., 2004**; **Timbermont et al., 2010**) or Annatto extracts (**Galindo-Cuspinera et al., 2003**)], organic acids (**Geier et al., 2010; Timbermont et al., 2010**),

enzymes (**Jackson et al., 2003**; **Engberg et al., 2004**), lysozyme (**Liu et al., 2010**),

Feeding "live" bacteria and yeasts

Supplementation of the broilers' diet with one or several beneficial bacteria has proven to be efficient to prevent the overgrowth of pathogens and the subsequent diseases. Several bacterial strains have been shown to increase broiler chickens performance (health, weight gain, feed conversion) and to prevent or reduce the incidence of diseases caused by pathogenic bacteria (**Patterson and Burkholder, 2003**; **Lutful Kabir, 2009; Chaucheyras-Durand and Durand, 2010**).

Probiotics

A probiotic is defined as "a live microbial food supplement that beneficially affects the host by improving the intestinal microbial balance" (**Fuller, 1999**). Indeed, probiotics can interact with the host to improve immunity and intestinal morphology or stimulate the metabolism, thus reducing the risk of infection by opportunistic pathogens. Probiotic bacteria have also been shown to produce molecules with antimicrobial activities, such as bacteriocins, that target specific pathogens, or even inhibit the adhesion of pathogens or the production of pathogenic toxins (**Joerger, 2003; Pan and Yu, 2014**).

Yeasts

Despite being under-represented in the literature as anti-*C. perfringens* agents, yeasts are known to have antimicrobial properties, which were recently reviewed (**Hatoum et al., 2012**). In addition the cell wall is, for many types of yeast, rich in beta-glucans, which have immunomodulatory properties (**Novak and Vetvicka, 2008**). On top of the beneficial effects they have on the host, yeasts can constitute a protection against pathogens by (i) producing mycocins, (ii) secreting enzymes that degrade bacterial toxins, (iii) preventing adhesion to epithelial cells, or (iv) by acting as a competitive exclusion agent (reviewed by Hatoum et al., 2012). For example, *Debaromyces hansenii* secretes molecules with anti-*C. butyricum* activity (**Fatichenti et al., 1983**), and *Saccharomyces boulardii* secretes a serine protease that inhibits the action of *C. difficile* toxins *in vivo* and *in vitro* (**Castagliuolo et al., 1996, 1999**).

Bacillus species

Several strains of *Bacillus* have been shown to have antagonistic activity against *C. perfringens* (Table (Table2).2). In most studies, the activity was linked to the production of bacteriocins. Indeed, within the *Bacillus* genus, several species are known to produce bacteriocins and antimicrobial peptides (**Stein, 2005**; **Lee and Kim, 2011; Mongkolthanaruk, 2012; Cochrane and Vederas, 2014**). For example, *B. thuringiensis* produces thuricin which is active against *C. difficile* (**Rea et al., 2010**).

The antagonistic species described in the literature include *B. cereus, B. licheniformis, B. pumilus*, and *B. subtilis*, which was the most represented. In a study involving over 200 *Bacillus* strains isolated from broiler feces, **Barbosa et al. (2005)** identified several species (*licheniformis, pumilus, subtilis*) with activity against *C. perfringens in vitro* (**Barbosa et al., 2005**).

Enterococci

A strain of *Enterococci faecium* (*E. faecium*) when fed to chicks on day of hatch was shown to reduce numbers of *C. perfringens* along with other pathogens after 28 days, and concomitantly to increase the counts of lactic acid bacteria (*Lactobacilli* and *Bifidobacteria*) (**Cao et al., 2013**). **Klose et al. (2010)** tested a number of *Enterococcus* strains, isolated from various animals, for their antagonism against *C. perfringens* and found that almost all had anti-*C. perfringens* activity, which could be attributed to the production of acids and hydrogen peroxide (**Klose et al., 2010**). *Enterococci* are known to produce a wide-range of bacteriocins, called enterocins, which are active against Gram-positive and Gram-negative bacteria (**Franz et al., 2007**). **Shin et al. (2008)** isolated a strain of *E. faecium* from broiler intestines that was active against *C. perfringens in vitro*, and identified the antimicrobial molecules as enterocins, with high homology to enterocins A and B (**Shin et al., 2008**).

Lactic acid bacteria

Lactic acid bacteria (LAB) are also very good probiotic candidates, as they display antimicrobial activities, but also have beneficial effects for the host. **Cao et al. (2012)** showed that adding *Lactobacillus fermentum* (*L. fermentum*) I.2029 to the diet of young chicks reduced the occurrence of *C. perfringens*-induced ileal lesions and inflammation. However, the effect on *C. perfringens* numbers was not measured in this study. Cao and colleagues also showed that the addition of the probiotic stimulated the host immune system, as seen by increased levels of cytokine expression, measured by real-time PCR (**Cao et al., 2012**). Many LAB isolates have exhibited anti-*C. perfringens* activity *in vitro*. For example, in a screening experiment involving 104 *Lactobacillus* strains isolated from geese feces, 84 strains were active against *C. perfringens* (**Dec et al., 2014**). Related to that, **Kim et al. (2007)** isolated several LAB (*Lactobacillus* and *Bifidobacterium*) from pig intestines that had antagonistic action against *C. perfringens*, with an *Lactobacillus amylovorus* (*L. amylovorus*) strain presenting properties amenable to be a potential probiotic candidate (**Kim et al., 2007**). The antimicrobial action of LAB is often attributed to the secretion of bacteriocins or the production of organic acids.

Competitive exclusion

The concept of competitive exclusion (CE) was originally described by **Nurmi and Rantala in 1973**, when feeding young chicks with bacteria isolated from a healthy adult chicken prevented colonization by *Salmonella infantis* (**Nurmi and Rantala, 1973; Rantala and Nurmi, 1973**). The exact mechanisms of action of CE remain unclear. However, it is now well known that implanting a "healthy" flora in the early days of life accelerates the establishment of the intestinal flora and creates a competition for nutrients within the intestine, thus preventing colonization by pathogens **(Joerger, 2003**; **Schneitz, 2005). Barnes et al. (1980**) described experiments in which 1-day-old chicks were fed caecal samples from healthy hens, containing, among others, several *Lactobacillus, Streptococcus faecalis, S. faecium* (now called *Enterococcus faecalis* and *Enterococcus faecium*) and *Bacteroides hypermegas* (Barnes et al., 1980). After 3 days, they observed a reduction in the number of *C. perfringens*, ranging from 100 to 1000 times lower in treated animals. Since then, several reports have been published, reporting a globally better intestinal health, a reduction in the number of *C. perfringens* and lower mortality, after administration of a CE product. The composition and efficacy of commercialized CE cultures have been the focus of several studies Elwinger and colleagues showed that the use of Broilact® reduced the mortality and occurrence of NE, with less *C. perfringens* in the caecum of animals in the treated group (**Elwinger et al., 1992**). CE cultures in association with a prebiotic containing essential oils and fructo-oligosaccharides (FOS) on chicks given an immunosuppressant vaccine, inoculated with *C. perfringens* and in dietary conditions favorable to NE development and led to reduced *C. perfringens* counts, a reduction in the intestinal lesions and lower mortality **(McReynolds et al., 2009).**

2. Molecules of microbial origin

Prebiotics

Prebiotics are additives that will stimulate the commensal flora and enhance the beneficial effects of probiotics within the host and are mostly indigestible oligosaccharides (**Patel and Goyal, 2012**). Numerous molecules have been described, with mannan-oligosaccharides (MOS) being the main prebiotic of microbial origin. MOS are components within the yeast cell wall and constitute the main active ingredient of yeast extract (YE) for disease control. They are often used as feed additives in broiler diets. where they have been shown to improve intestinal health and immune response, and also inhibit pathogen colonization by reducing adhesion. The addition of MOS to broiler feed was shown to improve overall performance as measured by productivity and weight gain **(Fowler et al., 2015)**. Thanissery and colleagues tested the effect of adding 2% yeast extract (NuPro, Alltech) to broiler feed, for the first 10 days of life, before a challenge with type A *C. perfringens* strains **(Thanissery et al., 2010).** Recently, **Abudabos and Yehia (2013)** tested another commercial yeast extract additive, Saf-Mannan, in a field trial for its ability to protect broiler chickens against NE. They performed a *C. perfringens* challenge on 16 days-old birds that were fed 0.05% Saf-Mannan since hatching, and compared their performance, gut health and *C. perfringens* counts on day 30. The chicks that were given the yeast extract showed overall better intestinal health (based on villi height) and had improved performance (measured by body weight gain and feed conversion ratio), which are consistent with the known beneficial effect of yeast extract on broiler performance. Moreover, the animals treated with Saf-Mannan had a 5 log reduction of *C. perfringens* numbers in the small intestine in comparison to the untreated animals **(Abudabos and Yehia, 2013).**

Bacteriocins

Bacteriocins are small ribosomally synthesized antimicrobial peptides that are produced by a large number of bacteria. They are classified based on their size, structure and post-translational modifications **(Cotter et al., 2013).** One of the main benefits of the use of bacteriocins is that some of them present a highly specific antimicrobial activity, so that they can be used to treat specific infections without altering the commensal gut flora. As discussed previously, the action of many probiotic strains is exerted through the secretion of bacteriocins (Table,2). Several examples of well-described bacteriocins with beneficial effects for broilers can be found. These include pediocin A, produced by *Pediococcus pentosaceus*, and divercin of *Carnobacterium divergens*, which were shown to improve broiler performance in a field trial (**Grilli et al., 2009; Józefiak et al., 2012)** as well as the well-characterized nisin produced by *Lactococcus lactis* that was shown to affect *C. perfringens* cells and spores *in vitro* (**Udompijitkul et al., 2012).**

Bacteriophages

Bacteriophages are highly species-specific viruses that infect and kill bacteria. Upon replication within the bacterial cell, phages produce endolysins, which target peptidoglycans and lyse the bacterial cell wall, freeing the phages and allowing them to spread to other cells (**Nakonieczna et al., 2015**). Phages were first discovered and described a century ago (**Twort, 1915**; **d'Hérelles, 1917**). Many bacteriophages of *C. perfringens* have been described and sequenced (**Morales et al., 2012**; **Seal et al., 2012; Volozhantsev et al., 2012**), including several that were isolated from strains of poultry origin and that

had specific anti-*Clostridium* activity (**Zimmer et al., 2002a; Seal et al., 2011; Volozhantsev et al., 2011; Seal, 2013**). The use of bacteriophages to limit *C. perfringens* infection has proven efficient in field trials. For example, **Miller et al. (2010)** showed that feeding broilers with a mixture of six bacteriophages reduced mortality in an NE challenge by over 90% and improved overall performance assessed as weight gain and feed conversion.

A number of studies focused on the use of bacteriophage endolysins as antimicrobials, rather than the phage itself **(Zimmer et al., 2002b**; **Tillman et al., 2013**; **Gervasi et al., 2014a; Swift et al., 2015**). The use of phage proteins instead of bacteriophages eliminates complications that can arise with phage therapy. Indeed, several studies have described bacteria becoming resistant to phage infection, by developing mechanisms to prevent the entry of the phage in the cell or by degrading the injected DNA **(Nobrega et al., 2015**). A purified recombinant endolysin of bacteriophage ϕ3626, isolated from a type strain of *C. perfringens*, was shown to have lytic activity against over 40 strains of *C. perfringens*, without affecting other *Clostridium* species or species of different genera, such as *Lactobacillus, Enterococcus* or *Bacillus* (30 and 34 strains tested respectively) **(Zimmer et al., 2002a,b)**. Recently, a modified endolysin was shown to be active against *C. perfringens* even at high temperatures, making it a suitable candidate as an antimicrobial additive for NE prevention **(Swift et al., 2015).** Another research group characterized the endolysin CP25L, isolated from a *C. perfringens* bacteriophage, which was active against *C. perfringens in vitro* (**Gervasi et al., 2013, 2014a**). The authors were able to over-express the enzyme in a modified *L. johnsonii* strain, strain which was discussed earlier in this review as active against *C. perfringens in vivo* (**La Ragione et al., 2004**; **Gervasi et al., 2014a,b).**

3. Vaccination against *C. perfringens*

A large number of trials tested the efficacy of broiler vaccination as a prophylactic treatment against *C. perfringens*-induced NE. For the purpose of this review, we will limit this section to an overview of the recent advances regarding vaccines against *C. perfringens*. The reader is also directed to a recent review by the Van Immerseel lab **(Mot et al., 2014).** Several strategies have been used to vaccinate broilers against *C. perfringens* to include use of live bacteria or inactivated toxins. Vaccines can be delivered by spraying chicks upon hatching, by addition to the feed or the drinking water, or even injected *in ovo* (**Sharma, 1999**; **Muir et al., 2000**; **Mot et al., 2014**). Vaccination using non-virulent *C. perfringens* strains have proven to be inefficient, and it has been shown that strains used in vaccines need to remain mildly virulent. **Thompson et al. (2006)** showed that strains with a mutation in the gene coding for the α toxin that were still virulent (but less than the wild-type) were able to protect chickens against NE, whereas an avirulent strain of *C. perfringens* did not have any immunizing effects. Several trials have shown that chickens could be protected against *C. perfringens*-induced NE by injection with inactive and active toxins **(Kulkarni et al., 2007**; **Jang et al., 2012**) and antigenic proteins (Jiang et al., 2009). Since the discovery of its role in NE, the NetB toxin has been intensively studied with regards to vaccination, with some promising results (**Fernandes da Costa et al., 2013; Keyburn et al., 2013a,b).**

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