



The Major Predisposing Factors for Necrotic Enteritis in Broiler Chickens and the Use of Probiotics as New Strategy to Prevent the Disease

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Abstract

Necrotic Enteritis (NE) caused by the pathogenic bacterium *Clostridium perfringens* is the most common and financially devastating bacterial disease in the poultry industry. The ban imposed by the European Union on the use of antimicrobial growth promoters to prevent and/or treat the NE has led to an increase in both the clinical and subclinical forms of the disease, causing a decrease in the production performances of chickens and, in the most severe cases, to an increase in mortality rate. Many predisposing factors can favour the colonization and proliferation of *C. perfringens*, threatening the health of birds. These predisposing factors include the alteration of gut microbiota, severe damage of the epithelial surfaces, and the alteration of the animals' immune status. Thus, the need for a prophylactic use of probiotics arises to prevent or mitigate the disease. Many bacterial species have shown probiotic activity, such as the genera *Bacillus*, *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*. These microorganisms, as well as favouring the colonization of the gastrointestinal tract by a beneficial microflora and promoting the correct development of chickens' immune system, they also compete with pathogenic bacteria to hinder their proliferation. This competitive exclusion mechanism exercised by probiotics promises to be an efficient and effective solution to replace the use of antibiotics in poultry industry, promoting animal health and favouring their productive performances.

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Introduction

The animal intestine is the perfect home for a multitude of microorganisms without which it is impossible to survive. For this reason, researchers consider animals as meta-organisms composed by the host and a complex microbiome. Gut microbiome that populates the Gastrointestinal Tract (GIT) plays a fundamental role in the development of the immune system, growth performance, use of nutrients, and protection against pathogens [1].

Necrotic Enteritis (NE) is one of the most common and, at the same time, financially devastating disease of broiler flocks. It is a multifactorial bacterial disease costing the poultry industry up to US\$ 6 billion per year in control measures and productivity losses [2]. The NE is an infectious disease caused by the species *Clostridium perfringens*, gram-positive, anaerobic bacteria found in soil, litter, dust, and, at low levels, in healthy birds' intestine. *C. perfringens* causes the disease when it con-

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verts from being a non-producer to a toxin producer, in fact, the NE is caused by the species that synthesize the NetB toxin, responsible for the formation of necrotic lesions within the intestine [3]. Although it has been confirmed that *C. perfringens* strains producing NetB toxin are the ultimate cause of the NE in chickens, it appears that simple infection is not sufficient to trigger the disease. Predisposing factors or risk factors are necessary to create a favourable environment for the proliferation of the pathogen [4,5].

The classic clinical form of the necrotic enteritis is characterized by a sudden increase in poultry mortality, up to 50%, often without any visible or warning sign, although wet litter can often be an indicator of the disease. The subclinical form of the infection is the most frequently encountered: There are no clinical signs of the disease, and usually not even a peak in mortality. Chronic lesions of the mucosa cause a loss in productivity due to impaired digestion and absorption, significant weight loss, and increase in feed conversion ratio [6,7]. Intestinal damage caused by subclinical infection can allow the bacteria to reach the biliary tract and portal bloodstream, eventually colonizing the liver and causing cholangiohepatitis [8]. Although the clinical manifestation of the NE can cause high levels of mortality, the subclinical form is the most important and harmful as it can persist in the flock without any type of manifestation. With no symptoms, chickens are not treated and, as a result, the subclinical necrosis causes great economic losses to poultry industry [9].

The disease causes gross lesions mainly to the small intestine, and less frequently to other organs, such as kidneys and liver. After the necropsy, the duodenum, jejunum, and ileum appear to have thinner-than-normal walls filled with gas. The clinical necrotic enteritis causes necrosis of large portions of the small intestine, covered with yellow-brown or bile-stained pseudo-membranes. The subclinical NE, on the other hand, presents ulcers on the mucosa surface, with discoloured and indefinite material that adheres to it [10,11]. Furthermore, the microscopic analysis of the late stages of the NE shows severe coagulative necrosis of the mucosa, mainly of the enterocytes of the intestinal villi [7].

Along with *Campylobacter* and *Salmonella*, *C. perfringens* is one of the most frequently isolated bacterial pathogens in foodborne disease outbreaks in humans. This confirms that *C. perfringens* infection in poultry poses a high risk of transmission via the food chain [12]. Given the high risk of bacteria developing resistance, the use of antibiotics has been banned in Europe and, considering the lack of a tested vaccine, alternative solutions need to be investigated to prevent *C. perfringens* infection in poultry. The use of probiotics could be a valid alternative to prevent chicken microbiota from being colonized by *C. perfringens*. Stanley et al. [13] demonstrated with a necrotic enteritis induction model via *C. perfringens* challenge, that the birds that developed the disease underwent significant changes in their microbiota compared to the birds that remained healthy. In this study, the group of animals that were administered a probiotic, specifically *Bacillus licheniformis* H2, and which were subsequently infected to induce the disease, developed less severe symptoms and a lower rate of change in microbiota composition than the birds not treated with the probiotic. This result confirmed the link between the microbiota composition and the outcomes of the disease [14].

Predisposing factors for NE development in chickens

The predisposing factors for necrotic enteritis in poultry are divided into four main groups (Figure 1): i) changes to the gut, ii) changes to bird immune status, iii) GIT microbiota disruption, and iv) pathogenic *C. perfringens* proliferation [5,7,14].

Changes to the gut. The first precaution to be taken to prevent *C. perfringens* infection is to avoid creating a favourable environment for pathogen proliferation. Diet thus becomes an important non-bacterial control factor that can influence the onset and incidence of the NE. Specific feed components can alter the digesta physical properties, promote *C. perfringens* growth, and even change GIT microbiota composition [5,7]. A diet rich in non-starch and poorly digestible polysaccharides is an important risk factor that predisposes the animals to the onset of the NE as, in addition to having a prolonged intestinal transit time, they increase the digesta viscosity, creating a favourable environment for the proliferation of *C. perfringens*. Therefore, wheat, oats, rye and barely are not recommended for poultry nutrition as birds fed with these grains are more likely to suffer NE than those fed with corn [15]. As previously mentioned, wet litter is a sign of possible *C. perfringens* infection: Diets rich in non-starch polysaccharides lead to an increased water ingestion, resulting in wet litter, which can consequently create a favourable environment to the contaminating sporulation of the pathogenic bacteria [5,15].

Coccidiosis infection is the best-known predisposing factor for NE. It has been shown that *C. perfringens* and *Eimeria* spp. Act synergistically to induce lesions typical of the necrotic enteritis [16,17]. *Eimeria* parasites colonize the small intestine and kill epithelial cells. The physical damage caused by the infection compromises the epithelial integrity of the GIT, and this could lead to serious consequences such as the opening of a direct access to the intestinal basal layer, the exposure of the extracellular matrix molecules, such as collagen, facilitating the adhesion of *C. perfringens*, as well as the over production of mucus which would provide another source of protein-rich nutrients for pathogen proliferation [5].

High dietary concentration of animal proteins, such as fish-meal, have also been found to be a risk factor for developing the NE. *C. perfringens* lacks many genes necessary for the biosynthesis of amino acids, so the bacteria can't grow in an environment where amino acids supply is limited. These diets contain poorly digestible proteins that remain in high concentration in the GIT, inducing *C. perfringens* growth and the consequent shift of the microbiota composition; these effects are modulated by the increase of nutrients, and probably also by pH increase throughout the gastrointestinal tract [18,5]. Knarreborg et al. [19] found that animal fat in diets, compared to vegetable fat, can also have effects on *C. perfringens* population. Finally, also the physical form of feed can influence the incidence of NE: Uniformly sized feed lowers the risk of disease occurrence compared to feed containing both large and small particles [20].

Changes to immune status. The moment of greatest risk for poultry to contract the NE is around the three weeks of age, when maternal antibodies begin to disappear from chicken's bloodstream. These major changes to the immune status cause an increased susceptibility to *C. perfringens* infection and proliferation [21].

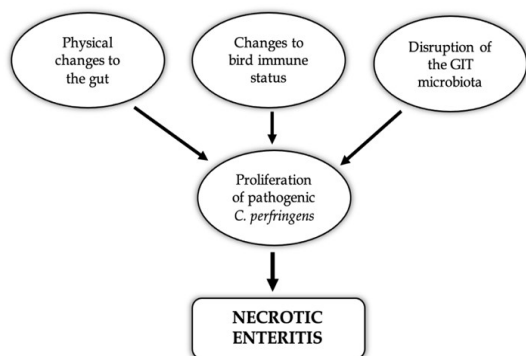


Figure 1: The four main factors for necrotic enteritis development in chickens.

Any type of stress can be considered as a potential risk factor that can predispose the animal to the onset of NE. Overcrowding, environmental ammonia, and physiological stress can lower the chicken's immune defences, exposing the animal to the potential infection caused by the pathogen. For this reason, it is recommended a not too high stocking density, as it could be a predisposing factor for contamination [22]. Furthermore, exposure to immunosuppressive agents, such as the viruses causing Marek's disease, Gumboro disease, or chick anaemia, reduce the animals' resistance to the infections of the GIT, increasing the severity of the NE [23].

GIT microbiota disruption. With recent advances in the investigative field of virology, it has been realized that there aren't low levels of the *C. perfringens* population within chickens' GIT which under certain predisposing circumstances proliferate to produce the disease, as previously believed, but these same strains, circulating at low levels in healthy birds, are actually non-pathogenic lines of the same species. Pathogenic strains appear to infiltrate and proliferate in a favourable environment at the expenses of non-pathogenic ones to dominate the *C. perfringens* population and thus induce the disease in birds [24,5]. Moore et al. [5] explain that groups of birds challenged with the pathogen and which develop the NE have significant differences in their gut microbiota compared to healthy birds. This underlines a causal relationship between the microbiota and *C. perfringens* colonization, although it's not clear whether it is the pathogen proliferation that induces changes to the bacterial flora or whether it is a certain type of microbiota that is more susceptible to infections and, consequently, to disease development [5].

Many of the factors that alter the physical state of the gastrointestinal system and the immunological state of the animal also affect the microbiota composition of the GIT. Recently, it has been shown that feeding animals with diets contaminated by *Fusarium's* mycotoxins, deoxynivalenol and Fumonisin (FBs), could be another predisposing factor for the onset of the necrotic enteritis [25]. It seems that, in addition to coccidiosis, FBs-contaminated feed also causes a reduction in the abundance of the Segmented Filamentous Bacterium (SFB) *Candidatus savagella*, which belongs to a unique group of commensal bacteria within the *Lachnospiraceae* family. Particularly present in the ileal mucosa of chickens, SFBs play an important role in modulating the host's immune system, especially in the most critical transition period from maternal and innate immunity to endogenous and adaptive immunity. It is precisely in this critical time window that chickens are most likely to contract the NE, suggesting the importance of further investigation on the role that SFBs may play in preventing or modulating the disease [25].

Lactobacillus is one of the predominant genera in the avian gastrointestinal system. These bacteria are important for the role they play in the induction of immunomodulation and for the protection they offer with their antagonistic activities against pathogens. The administration of fishmeal-based diets or diets contaminated with FBs have been shown to induce changes in the composition of lactobacilli species within the caecum of chickens, without changing the total count. In particular, *Lactobacillus johnsonii* and *Lactobacillus acidophilus* suffer a drastic decrease, while the abundance of *Lactobacillus reuteri* and *Lactobacillus animalis* increases. The same changes were observed in broilers challenged with the pathogenic strain *C. perfringens*. Some lactobacilli, such as *L. johnsonii*, are of great importance for their probiotic activities, including the inhibition of pathogens [26]. They ferment carbohydrates introduced through diet into lactic acid as major final product, which lowers the intestinal pH causing the inhibition of the growth of acid-sensitive bacteria [27]. Furthermore, the role that lactobacilli play in a cross-feeding process is fundamental: Through the production of lactate, they promote the activity of butyrate-producing bacteria.

Butyrate is an important signal molecule of the GIT and it is also an anti-inflammatory metabolite that participates in the stabilization of intestinal integrity, the improvement of the productive performance of the animal, the change of microbiota composition, and the metabolic activity of the entire microbial system in the gut. The use of fishmeal in animal diets and the administration of *Eimeria* have shown being two of the main causes of the abundance's decrease of the *Ruminococcaceae* and *Lachnospiraceae* populations, the main butyrate-producing bacteria in the intestinal system of chickens and mammals [28,29]. Butyrate has been repeatedly shown to reduce the incidence of severity of necrotic enteritis when administered as additive. Their mode of action is not yet clear but, by colonizing mainly the caecum of the chickens, butyrate-producing bacteria can suppress the pathogenic *C. perfringens* in the caecum, preventing the ascent of the infection. Thus, the signalling function of butyrate make it an essential metabolite for the universal protective mechanism in all animal species [25,30].

Pathogenic *C. perfringens* strains proliferation. Non-pathogenic *C. perfringens* strains are frequently isolated from healthy broilers, while only one type of strain predominates in poultry affected by the NE. It isn't yet clear whether the chickens that develop the disease already have the pathogenic strain within their GIT that proliferates under favourable conditions, or whether certain factors cause the bacteria introduction into the organism. As previously explained, many factors can cause the proliferation, but it is important to understand how the selective proliferation of pathogenic *C. perfringens* occurs compared to non-pathogenic strains [5]. Timbermont et al. [24], discovered that the antimicrobial protein perfrin, metabolized only by pathogenic strains, inhibits the growth of commensal *C. perfringens* strains. It is therefore probable, but still not confirmed *in vivo*, that the pathogenic strain could produce bacteriocins that inhibit the growth of closely related strains, thus favouring its selective proliferation.

Alpha toxin was initially thought to be the major virulence factor of NE in broilers, despite both pathogenic and non-pathogenic type A strains produce it. Yet another study disproved this assumption, showing that alpha toxin was unrelated to lesions caused by the disease [31]. Recently, a new toxin associated with NE in broilers has been discovered, the *C. perfringens*

necrotic enteritis B-like toxin (NetB), which is a member of the β -barrel pore-forming toxin family. With an *in vitro* study, Keyburn et al. [3] demonstrated that, in addition to forming pores in plasma membrane, the toxin causes cell rounding and lysis in chicken male hepatoma cell line. NetB appears to be an efficient environmental adaptation, as it is produced when *C. perfringens* concentration is high and nutrient availability is limited. The damage that the toxin causes to the host cells provides enough nutrients for the bacteria to survive [32]. The discovery that the netB gene encoding for NetB toxin is carried on a conjugative plasmid suggests the possibility of exchange the plasmid between different *C. perfringens* strains, and consequently the potential transformation of a non-pathogenic strain into pathogenic [5,33].

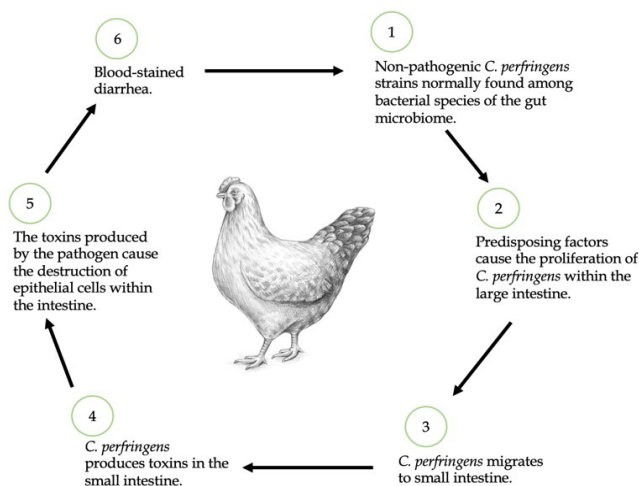


Figure 2: The pathogenesis of necrotic enteritis caused by the proliferation of the pathogenic *C. perfringens* and by the production of the toxins that destroy the intestinal epithelial cells, lead to blood-stained diarrhea typical of the disease.

Prophylactic use of probiotics to prevent or mitigate NE

The use of Antimicrobial Growth Promoters (AGPs) has been a standard practice in poultry industry for years. AGPs have been used to increase broilers' weight and also to control the occurrence of necrotic enteritis and other diseases. In 2006, the European Union decided to ban their use to avoid the danger of developing resistance in bacterial strains, so the NE outbreaks began to be more frequent. Therefore, great efforts are still underway to find new strategies to prevent or mitigate the symptoms of the disease.

Probiotics are defined as live non-pathogenic and nontoxic microorganisms, which when administered through the digestive route, are favourable to the host's health (Figure 3) [34]. There is an unstable balance between beneficial and non-beneficial bacteria in the normal GIT of healthy chickens. When this balance exists, the birds show optimal performances but, in stressful conditions, the beneficial flora decreases in number, allowing non-beneficial bacteria to grow. This can predispose animals to frequent diseases, or even reduce growth parameters and feed efficiency. Probiotics reduce the risk of developing the NE by improving the animal's immunity, the balance of intestinal microflora, and by stimulating the host's metabolism. Probiotics, in addition to competing with pathogenic bacteria for the available nutrients, they also produce microbial substances that inhibit their growth and proliferation [35]. Several strategies have been proposed to understand the mechanism of action of probiotics. These include the modulation of the immune system,

action on bacterial virulence factors, interference on bacteria-induced signalling pathways, competition for nutrients, production of organic acids and bacteriocins, anti-inflammatory capacity, and inhibition of pathogen adhesion to the epithelium [36].

The composition of the microflora of broilers' GIT plays a fundamental role in preventing the onset of the necrotic enteritis. As previously discussed, butyrate-producing bacteria have the ability to inhibit the inflammation of the intestine and preserve its normal activity. Lactic-acid producing bacteria, through a cross-feeding mechanism, promote gut colonization by butyrate-producing bacteria. Similarly, the filamentous bacteria *Candidatus savagella* induces the formation of immunoglobulin A. Many other microorganisms have shown the ability to counteract the proliferation of *C. perfringens*, such as the genera *Bacillus*, *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and yeasts. Therefore, all these microorganisms can be considered as potential probiotics [37].

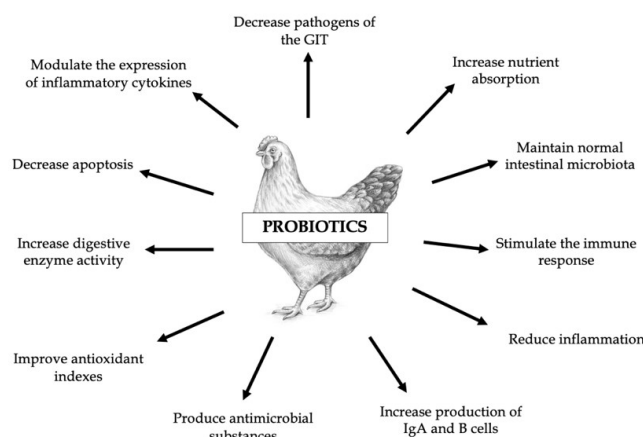


Figure 3: Beneficial effects of probiotics on broiler chickens health.

Many species of the genus *Bacillus* have shown anti *C. perfringens* activity, including *B. licheniformis*, *B. pumilus* [38], *B. subtilis* [39], and *B. cereus* 8A [40]. *Bacillus* species promote the growth of NE-affected chickens by improving their feed efficiency and facilitating weight gain. Furthermore, as well as regulating fatty acid synthesis and the genes related to sick chickens' liver oxidation, they also enhance the antioxidant activity [41]. *Bacillus* spp. produce bacteriocins that inhibit the growth and proliferation of *C. perfringens*, and their spores are used in feed for their ability to increase chickens' productive performances and to reduce the mortality of the birds affected by NE [42]. When used as probiotic, *B. subtilis* improves the microbial balance in chickens' GIT by stimulating the immune system and competing for the exclusion of pathogenic strains. These bacteria have extensive activity against *C. perfringens*, they can decrease pathogen's abundance in the ileum and caecum by altering the intestinal microflora composition of the host and by supporting the improvement of broilers' growth performance [37]. Al-Baadani et al. [43] showed that chickens challenged with *C. perfringens* and fed a diet enriched with *B. subtilis* as probiotic, not only had a decrease in triglycerides and total cholesterol in serum, but also a significant increase in lymphocytes number, thus demonstrating the potential of these bacteria as probiotic for the prevention of necrotic enteritis. Another species, *B. licheniformis*, when administered in diets, improves the growth performance and alters gene expression of lipid and fatty acid metabolism. Moreover, *B. licheniformis* also improves

the balance of the microflora of the ileum and caecum in NE-affected poultry [44].

The antibacterial properties of lactic acid producing bacteria allow their use for the prevention of the NE. Their probiotic potential is attributed to their ability to produce lactate, bacteriocins, hydrogen peroxide, and enhance host immunity by increasing cytokine expression. There are many species of *Lactobacillus* that can be used as probiotics, and these include *L. acidophilus*, *L. animalis*, *L. fermentum*, *L. johnsonii*, *L. mucosae*, *L. plantarum*, *L. reuteri* and *L. salivarius* [37]. *L. johnsonii* has the ability to inhibit *C. perfringens* colonization and consequently prevent NE. The administration of this strain to chickens affected by the disease reduces daily weight loss, improves lipid metabolism and intestinal microflora, and mitigates liver abnormalities. Therefore, its use as probiotic can be of great advantage in preventing or alleviating the symptoms of necrotic enteritis [45]. *L. acidophilus* inhibits the growth of pathogenic bacteria in the gut and modulates the immune system of the birds. Its probiotic activity improves the efficiency of weight gain and significantly decrease flock mortality [46].

The ability of *Enterococcus* to produce hydrogen peroxide and bacteriocins with anti *C. perfringens* activity, and to inhibit the production of toxins, makes them a potential powerful probiotic for the prevention of NE. In chickens challenged with the pathogenic strain, the administration of *Enterococcus faecium* helps to alleviate weight loss, intestinal injury, histopathological inflammation and prevents the apoptosis of intestinal cells [37].

The importance of a founding microflora in broiler chickens

The industrialization of chickens production has transformed their microbiota to such an extent that it is no longer possible to associate it with that of their ancestral precursors due to the un-natural practices whereby chicks are separated from their mothers before hatching. Thus, chicks are immediately exposed to completely different bacteria from those of the bacterial communities historically found in chickens' gut and adapted to their host [47]. In commercial practices of poultry industry, newly hatched chicks are exposed to the microbes deriving from the hatchery environment, human handlers, transport boxes, and transport vehicles, before arriving at the farm. This process is generally carried out within the first hours of life, moment in which there is a rapid increase in bacterial diversity and bacterial load in chicks' gut. These first bacterial sources have a significant influence on the development of the intestinal microbiota as it is during these first hours of life that the most significant colonization of chickens' intestine takes place. Thus, the absence of the natural environment and contact with adults of the same species makes the chicks' GIT founding community particularly susceptible to environmental and human bacterial sources [48]. The growth of a bacterial community in chicks is very rapid. Only one day after hatching the ileum can contain up to 10^8 bacteria per gram, and the caecum 10^{10} bacteria per gram. These numbers grow exponentially day by day and continue to adapt to the environmental changes and to the potential stressors that can threaten the host's immunity [49,47].

Initial bacterial colonization inhibits or promotes the invasion and stabilization of subsequent bacteria in the intestinal environment and produces metabolites that can support or retard the growth of other bacteria. Therefore, the first days post-hatch are essential for the efficient colonization by beneficial bacteria and the selective exclusion of pathogenic ones [5]. The type of diet consumed by chickens has an important

impact on the composition of their gut microbiota, as it provides the nutrients that the bacteria can use before or after being processed by the host. Several studies have shown the importance of feeding chicks immediately after hatching as it increases the animals' productive performance, helps them to grow efficiently, and drastically decreases the mortality rate [19,20,35]. It follows that, as well as the immediate administration of essential nutrients for chicks' growth, a good strategy to ensure the development of an efficient immune system is to administer probiotics that can contribute to forming a founding population of commensal bacteria with the ability to selectively exclude the pathogenic strains, including *C. perfringens* [26,34]. Researchers confirm that the competitive exclusion produced by inoculating an adult microflora in day-old chicks has a positive impact on the intestinal function and disease resistance. This approach allows to provide the chick with a complete adult microbiota, thus avoiding having to add one or more bacterial strains to an already-formed microbiota [36,37]. Given the high chicks' susceptibility to infections, this practice has a very important commercial value.

Conclusion and future perspectives

Necrotic enteritis caused by the bacterium *Clostridium perfringens* is the cause of huge economic losses in terms of production performance in poultry industry, especially after the European Union's ban on the use of antimicrobial growth promoters to prevent or treat the disease. Cost-efficient alternatives are therefore sought to urgently remedy the frequent outbreaks of the NE in chicken flocks. First of all, among all the studies carried out, it is necessary to find a consensus and reproducible experimental model in which all aspects, including the feed composition and the prevention of the predisposing factors, are standardized to allow further progress in the development and evaluation of new strategies to efficiently control the spread of necrotic enteritis among birds. A combination of measures aimed to avoid the predisposing factors and to fight the pathogen, appears to be the best strategy to adopt. Probiotics may be the best alternative to replace the use of antimicrobial growth promoters to relieve intestinal inflammation caused by *C. perfringens*. Many microorganisms have shown to have efficient probiotic activity, including the genera *Lactobacillus*, *Bacillus*, *Bifidobacterium*, and *Enterococcus*. However, it is commonly believed that the beneficial effects of these probiotics should be investigated under real farm conditions to better understand their mode of action. Therefore, further studies are needed to define the colonization mechanism of *C. perfringens*, and to develop new probiotics that can completely replace antibiotics to hinder the pathogenic bacterium growth into the host and thus prevent the onset of the necrotic enteritis.

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