Clostridium perfringens-Induced Necrotic Diseases: An Overview

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Abstract: *Clostridium perfringens*, a prevalent Gram-positive bacterium, causes necrotic diseases associated with abundant life loss and economic burdens of billions of USD. The mechanism of *C. perfringens*-induced necrotic diseases remains largely unknown, in part, because of the lack of effective animal models and the presence of a large array of exotoxins and diverse disease manifestations from the skin and deep tissues to the gastrointestinal tract. In the light of the advancement of medical and veterinary research, a large body of knowledge is accumulating on the factors influencing *C. perfringens*-induced necrotic disease onset, development, and outcomes. Here, we present an overview of the key virulence factors of *C. perfringens* exotoxins. Subsequently, we focus on comprehensively reviewing *C. perfringens*-induced necrotic diseases such as myonecrosis, acute watery diarrhea, enteritis necroticans, preterm infant necrotizing enterocolitis, and chicken necrotic enteritis. We then review the current understanding on the mechanisms of myonecrosis and enteritis in relation to the immune system and intestinal microbiome. Based on these discussions, we then review current preventions and treatments of the necrotic diseases and propose potential new intervention options. The purpose of this review is to provide an updated and comprehensive knowledge on the role of the host–microbe interaction to develop new interventions against *C. perfringens*-induced necrotic diseases.

Keywords: enteritis; immunity; exotoxin; host microbiota interaction; metabolite

1. Overview of *C. perfringens* Produced Toxins and Enzymes

Clostridium perfringens, a Gram-positive and spore-forming anaerobic bacillus of the genus Clostridium, ubiquitously resides in nature of animal microbiota, decaying plants, soils, and marine sediments. *C. perfringens* has induced a large array of severe diseases over the centuries in muscle, gut, and other organs/tissues, such as gas gangrene (Clostridial myonecrosis), foodborne or non-foodborne poisoning of diarrhea, necrotizing enteritis, enterotoxaemia, and necrotic enteritis. Besides severe life-threatening complications and numerous mortalities, it is estimated that *C. perfringens*-induced diseases cause USD 0.2–1.7 billion yearly in human foodborne enteritis in the USA alone [1] and USD 6 billion yearly in the poultry industry around the world [2]. The successful induction of different diseases, in part, comes from *C. perfringens*' ability to produce more than twenty pathogenic toxins and enzymes in various combinations [3]. *C. perfringens* strains are currently classified into seven toxin types (A to G) based on their ability to produce various combinations of six major toxins, namely alpha (CPA), beta (CPB), epsilon (ETX), iota (ITX), enterotoxin (CPE), and enteritis B-like toxin (NetB) (Table 1) [4,5]. In the laboratory, *C. perfringens* is often cultured at 37 °C anaerobically with Cooked meat medium [6], Duncan-Strong sporulation medium [7], Fluid thioglycolate medium (FTG) [8], Tryptic soy broth (TSB) [9], Tryptose-sulfite-cycloserine (TSC) agar [10], and TSA with egg yolk emulsion or 5% sheep blood [11]. *C. perfringens* is provisionally identified as a nonmotile and Gram-positive bacillus, producing black colonies on TSC agar, reducing nitrates to nitrites, producing

acid and gas from lactose, and liquefying gelatin within 48 h [12]. It has a very short generation time at 6.3 min in fluid thioglycolate medium (FTG) under 43 °C and anaerobic condition [13].

Table 1. Main toxins produced by *C. perfringens* and their locations.

Toxins	Toxin Types	Encoded Genes	Gene Location	Protein Size, kDa
Alpha (CPA or PLC)	A, B, C, D, E, F, G	*cpa* or *plc*	Chromosome	42.5
Beta (CPB)	B, C	*cpb*	Plasmid	35
Epsilon (ETX)	B, D	*etx*	Plasmid	34
Iota (ITX)	E	*iab* and *iap*	Plasmid	Ia: 47.5, Ib: 71.5
Enterotoxin (CPE)	C, D, E, F	*cpe*	Chromosome or Plasmid	35.5
Enteritis B-like toxin (NetB)	G	*net*B	Plasmid	28

Most *C. perfringens* isolates produce α-toxin (CPA or PLC), a multifunctional zinc metalloenzyme comprising of phospholipase C and sphingomyelinase [14]. CPA toxin is encoded by the *plc* (or *cpa*) gene that is located on the chromosome and is the major virulence factor for gas gangrene in human and animal (clostridial myonecrosis) [15]. In the progression of gas gangrene, CPA penetrates the cell membrane with holes and activates the arachidonic acid cascade and protein kinase C, resulting in cellular disfunction [14,16]. Meanwhile, CPA suppresses the immune response by restraining leukocytes from entering the infected tissues [17] and reduces the blood supply to infected sites by triggering vasoconstriction, thrombosis, and platelet aggregation [14].

CPB toxin is produced by types B and C strains of *C. perfringens* and the gene (*cpb*) encoding CPB is located on the virulent plasmid [18,19]. This plasmid also carries other toxin genes such as toxin perfringens Large (*tpeL*) or *cpe* [20]. At the cellular level, CPB causes necrosis and hemorrhage in the small and large intestines [3,21], and resulting numerous diseases in humans and animals [15]. For instance, *C. perfringens* type B induces the fatal hemorrhagic dysentery in sheep, while the type C bacterium causes human enteritis necroticans (Pigbel), necrotizing enteritis, and enterotoxaemia [15,22,23]. It is reported that CPB is also responsible for mouse enterotoxemia [22].

ETX toxin encoded by the *etx* gene on the plasmid is mainly produced by *C. perfringens* types B and D [23–25], which are responsible for enterotoxemia in goats and sheep and less frequently in cattle [26,27]. This toxin also targets organs besides the intestine, such as the heart, the central nervous system, and the lungs [4], shown as severe brain lesions, hemorrhages, necrosis of the white matter, and vascular edemas [4].

ITX toxin, which is encoded by two separate genes *iab* and *iap* on large conjugative plasmids, is produced by *C. perfringens* type E strain [28]. ITX causes cells necrosis by inducing cytotoxic activity, which affects mitochondrial function, ATP reduction, increased inorganic phosphate (IP), and cells swelling [29], and the toxin is responsible for animal diseases such as hemorrhagic enteritis [28].

The *C. perfringens* Enterotoxin (CPE) is a 35.5 kDa polypeptide [30] and detected in type C, D, E, and F strains [31]. CPE is encoded by the *cpe* gene located on either plasmids or chromosome among 5% of type A stains. Moreover, this toxin is only expressed during *C. perfringens* sporulation. It is accumulated in the beginning of the sporulation and excreted out when cells lysate at the end of sporulation [32,33]. The *cpe* gene is found in *C. perfringens* type E strain as a silent gene [34,35]. The CPE is highly related to food poisoning in humans [36].

Another common toxin that produced by *C. perfringens* type G strain [4] is Necrotic Enteritis B-Like Toxin (NetB). NetB is encoded by the *netB* gene that is located on large and conjugative plasmids [37,38]. This toxin causes cell lysis by osmotic pressure and cell necrosis, inducing avian necrotic enteritis under certain predisposing factors [2,39]. Table 1 summarizes *C. perfringens* toxins and gene locations.

C. perfringens also produces other toxins and enzymes to degenerate tissues, including Theta (θ) toxin, κ toxin (collagenase), proteases, hemolysins, μ toxin (hyaluronidase),

ν toxin (deoxyribonuclease), and neuraminidase [40]. θ toxin weakens the host inflammatory response to the infection by destroying blood vessels (hemolysis), breaking down leukocytes, and leading to cardiotoxicity [40,41]. κ toxin helps the bacterium's rapid spread from connective tissue plains into deeper muscle tissues by breaking down connective tissue [42]. Although μ toxin is a non-lethal toxin, it has the ability to facilitate the spread of α toxin and degrades mucins and connective tissues [43]. ν toxin displays haemolytic and cytotoxic activities, and damages the nuclei of polymorphonuclear leukocytes and muscle cells in gas gangrene [44]. These toxins and proteins work together to cause necrosis of muscle, subcutaneous tissue, and add in the production of gas with hydrogen sulfide and carbon dioxide.

2. Overview of *C. perfringens*-Induced Diseases

Unlike other anaerobic bacteria infecting limited animal hosts and their tissues, *C. perfringens* enjoys a successful living spectrum from muscles to the gut (Figure 1). *C. perfringens*-induced diseases show complex manifestations of rapid bacterial overgrowth [45–47], rapid gas accumulation [48,49], collateral inflammatory self-destruction [9], and various toxin productions [50,51]. At a glance, these diseases seem loosely related, but they share the same pathogen of *C. perfringens*, and often with the same toxins. In the following, we briefly review up-to-date progression on understanding of the important *C. perfringens*-induced necrotic diseases.
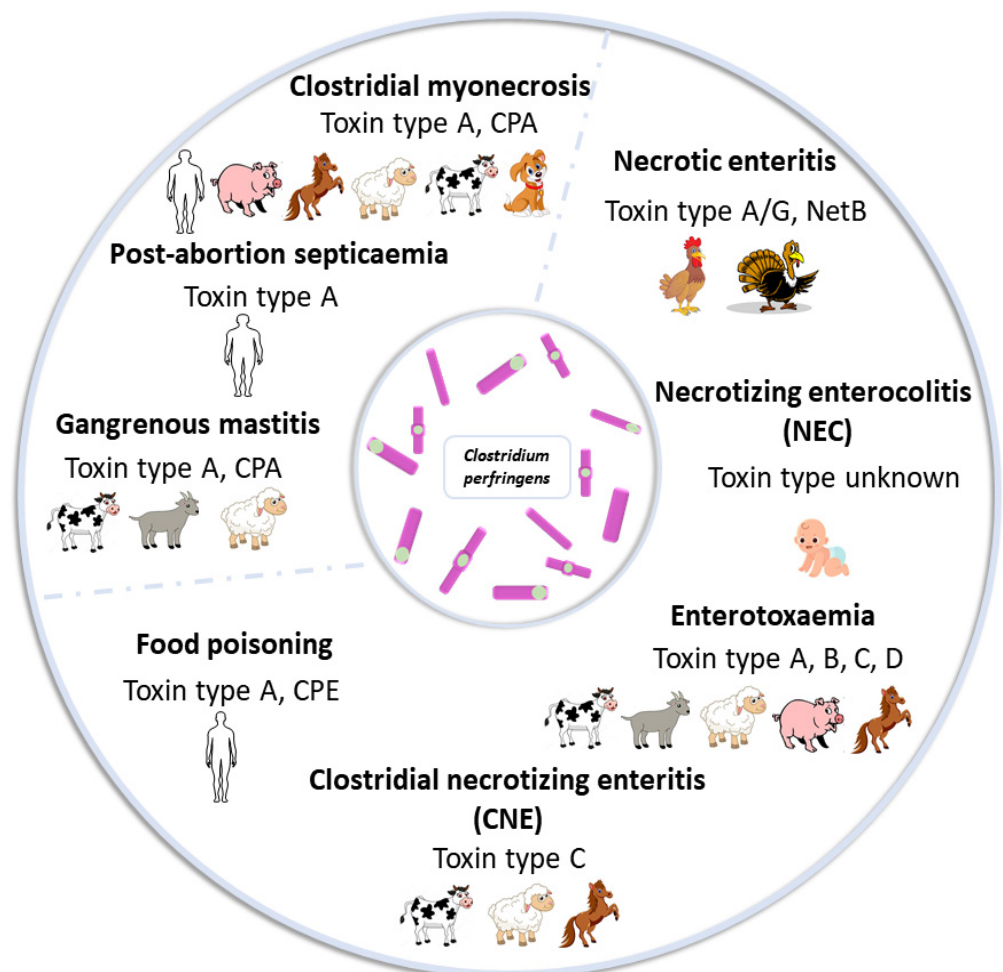

Figure 1. Overview of *C. perfringens*-induced diseases.

2.1. C. perfringens-Induced Necrotic Diseases in Wounded Soft Tissue

Clostridial myonecrosis (gas gangrene) is a highly lethal necrotizing soft tissue (muscle) infection characterized by skeletal muscle necrosis, emphysema, subcutaneous edema, multiple organ failures, and high mortality [20]. Gas gangrene is classified into two types, traumatic gangrene and spontaneous gangrene. *C. perfringens* is responsible for 80–90% [52] of traumatic gas gangrene in both humans and animals, while *C. septicum* causes spontaneous gangrene [53]. Around 1000 to 3000 human cases are reported in the USA yearly [54], while it is estimated that the prevalence rate is higher in developing countries where a functional healthcare system is lacking. In most cases, the onset and clinical course of gas gangrene in traumatic areas is rapid from hours to days if the wound is infected with a sufficient number of *C. perfringens* [55]. Gas production and myonecrosis are the main markers, while other signs may be visible within a few days, such as the formation of blackened sores on the skin, black bubbles with crepitus, foul smell in decomposing tissues, and limb edema [52]. With the rapid systemic spread of *C. perfringens* and its toxins, an overall mortality of 20–30% [56,57] is followed with the systemic complications, such as tachycardia, fever, swelling, jaundice, disseminated intravascular coagulation, toxemia, hemodynamic shock, hypotension, renal failure, peritonitis, and sepsis [57]. *C. perfringens* induces gas gangrene mainly through the production of CPA. Meanwhile, the traumatic injuries in the deep tissues provide a favorable environment with abundant amino acids and peptides, as well as lower oxygen tension for the anaerobic bacteria's rapid growth and secretion of extracellular cytolytic toxins for further nutrient release from the host tissue [55]. Several animal models have been developed to understand the pathogenesis of *C. perfringens*-induced gas gangrene. For example, guinea pigs pretreated with either mechanically macerated muscles or with applied $CaCl_2$ injections prior to *C. perfringens* infection were used to mimic human gas gangrene [53]. Stevens and his colleagues induced gas gangrene in a Swiss Webster mice model with 10^9 colony forming units (cfu) of *C. perfringens* cells injected into the right upper thigh muscles [58]. Although not as frequently valued as in traumatic and gastrointestinal surgery-related myonecrosis in humans, *C. perfringens*-induced gas gangrene is also a highly lethal histotoxic disease that affects several animal species, including sheep, cattle, horses, goats, pigs, and dogs [59–62]. It is reported that Clostridial myonecrosis in horses is not only associated with wounds or lacerations, but also followed by intramuscular injections or the inadvertent administration of drugs around neck musculature [63]. It is often accompanied with autoimmune hemolytic anemia, severe intravascular hemolysis, and hemoglobinuria [64].

Besides gas gangrene, *C. perfringens* also occasionally induces other soft tissue diseases, such as gangrenous mastitis in cattle [10], goats [11], and sheep [65]. The disease is characterized by severe inflammation in the mammary glands, discolored udders, watery milk containing abscesses and draining pus, and/or blood secretions. Systematic fever, pneumonia, septicemia, toxemia, and death may occur [11]. Furthermore, *C. perfringens* occasionally causes human maternal sepsis after abortion, called post-abortion septicemia. A high mortality rate is followed with abdominal pain, vaginal bleeding, and gas formation in the endometrial cavity [13,14].

2.2. C. perfringens-Induced Enteritis with Tissue Necrosis

Besides infecting wounded soft tissues, *C. perfringens* is also able to affect the gut system. As we know, *C. perfringens* induces foodborne or non-foodborne diarrhea. It is one of the top five causes of food poisoning in the United States, alongside norovirus, *Salmonella*, *Campylobacter*, and *Staphylococcus aureus* [66]. Nearly one million *C. perfringens*-induced food poisoning cases are reported yearly in USA [1], which results in an economic loss ranging from USD 22 million to USD 1.7 billion yearly in US. The enteritis is developed when people accidentally ingest a sufficient number of the bacterium (10^6 cells/g food) [67]. Survey shows most of the population has experienced subclinical food poisoning of *C. perfringens* as they have antibodies against its toxin [68]. Contaminated meat or poultry that is not fully cooked or stored improperly for a long time is the common source of harboring the

bacterium. Typical signs and symptoms of abdominal cramping, pain, diarrhea, nausea, vomiting, and fever usually begin 8–12 h after exposure to the bacterium [69]. A complete remission may be within 24 h, but for patients with poor health conditions, it can last up to 2 weeks [70]. The type A strain, which can produce *C. perfringens* enterotoxin (CPE), is the main strain related to human food poisoning in developed countries. At the cellular and molecular levels, CPE binds to claudins on epithelial cells and forms many pores in the cell membrane, leading to an influx of Ca^{2+} into cells [71], unbalanced ions, and apoptotic, or oncotic death [72]. CPE is a heat-sensitive protein and is inactivated at 165 °F [73]; thus, cooking with high temperatures and thoroughly is a way to avoid infection. Because *C. perfringens* is able to form spores in unfavourable conditions, the infective bacterium may revive in food fully cooked but left standing for a long time. To prevent the food poisoning, food, especially meat, should be cooked thoroughly to recommended temperatures, and food leftover should be stored in the refrigerator and reheated to above 140 °F before consumption [74].

Clostridial necrotizing enteritis (CNE) is another type of food poisoning and has a high fatality. CNE mainly occurs in patients lacking trypsin, and the main causative strain is type C that secretes β toxin [75]. Other factors also trigger CNE, such as poor hygiene and consuming trypsin inhibitor-harboring food, or food infected with ascaris that can secrete trypsin inhibitor [76]. CNE shows various signs of inflammation, perforation, and necrosis in jejunum and ileum as well as symptoms of diarrhea, abdominal pain, bloody vomiting, hemorrhaging, septic shock, and peritonitis [76,77]. CNE is rare in USA, but it has relatively higher prevalence in other countries such as Central America, South America, Africa, Asia, and New Guinea [78].

Necrotizing enterocolitis (NEC) is a leading cause of gastrointestinal morbidity and mortality in premature infants, whose birth weight is less than 1.5 kg [79]. Clinical signs differ from baby to baby, but normally include ileitis, abdominal distention, green fluid in the stomach, hematochezia, as well as lethal symptoms of apnea, metabolic acidosis, shock, and disseminated intravascular coagulopathy [80]. It is believed that NEC could be related to the pathogen infection [81] as *C. perfringens* can be detected in some stool samples of the NEC patients. The nonspecific clinical symptoms make it difficult to build a suitable animal model as well as underline the etiology of NEC. However, prematurity, initial microbial colonization, and using formula instead of breast milk feeding are considered to play a pivotal role in the development of NEC [82,83].

Necrotic enteritis (NE) is estimated to be responsible for around USD 6 billion losses to the poultry industry every year [2]. The increased NE incidence is associated with the trending restriction of prophylactic antimicrobial supplementation in farm animal production in the absence of effective alternatives [84]. NE is an acute enteric disease that primarily affects 2–5 week-old broiler chickens and 7–12 week-old turkeys, characterized by patches of necrotic tissue on the intestinal epithelium [85]. Severe depression followed by a suddenly increased mortality is usually seen in clinical NE, while bloody diarrhea, dehydration, ruffled feathers and inappetence, body weight loss, and production decrease are more often in subclinical cases [85,86]. *C. perfringens* type A and G are the main causative agents of chicken NE according to bacteria distribution and field isolation in broiler chickens [87,88]. However, the pathogenesis of this bacterium on NE is not totally clear. The successful chicken disease model always needs predisposing factors to be present, including coccidiosis infection, stress, dietary factors (mycotoxin-contaminated feed, high protein feed), etc., which may result in the damage of epithelial tissue, an increase in mucus production, the release of amino acids and nutrients, and the disruption of gut resident microbiota. These factors in turn may facilitate *C. perfringens* growth and toxin release. Two main NE chicken models have been developed to mimic NE outbreak in poultry industry. Co-infection with *Eimeria* (in particular *E. maxima*) and *C. perfringens* highly resembles NE in poultry farms because field NE outbreak is associated with *Eimeria*, and immunized birds with the *Eimeria* vaccine greatly reduces the risk of NE incidences [8,9]. This co-infection model has a predisposing factor of injury

and inflammation by *Eimeria* infection. The second NE model is *C. perfringens* infection alone, often with dietary manipulation, such as a high fishmeal diet [89], a wheat-rye diet, and others [90]. Together, the results from these two models need to be carefully interpreted based on the context. Alpha toxin in *C. perfringens* type A strain has been regarded as the primary virulence factor for NE in poultry [91,92]. However, there is no significant difference between the level of alpha toxin produced by strains associated or not with NE development [92]. Using *C. perfringens* infection alone as a model, a high level of alpha toxin in the intestinal tract does not inevitably lead to NE disease [93]. On the other hand, the null mutation of alpha toxin in a chicken NE-isolated EHE-NE18 strain does not affect the bacteria's pathogenicity in following chicken experiments [94]. NetB is another extensively studied toxin considered to contribute to NE progress, which belongs to the α-haemolysin family of β-pore-forming toxins and has a 38% similarity to *C. perfringens* β-toxin [95]. This toxin forms pores in the plasma membrane and causes cell rounding and lysis in chicken Leghorn male hepatoma cell lines through a polyethylene glycol (PEG) block experiment [96]. NetB inducing NE is determined in a *C. perfringens* infection alone model [95]. Epidemiologically, *C. perfringens* isolates carrying netB genes in chickens with NE outbreak is higher than in *C. perfringens* isolated from healthy chickens [97–99]. However, opposite survey results were reported by Abildgaard [100]. NetB-positive strain colonizing in healthy birds suggests that *C. perfringens* or NetB toxin alone is not sufficient to induce the disease.

C. perfringens-induced enteritis are also categorized as enterotoxemia with an acute poisoning condition induced by the toxins. This lethal disease can affect cattle [101], lambs [102], sheep, goats [103], and many other animals [103]. Almost every type of *C. perfringens* is involved in enterotoxemia. As we mentioned above, *C perfringens* type A strain is a part of the normal intestinal microbiota of animals, which is mostly responsible for foodborne or nonfoodborne intestinal diseases. It is also implicated in the enterotoxemia of domestic animals [29]. Enterotoxemia caused by type A in lambs is known as yellow lamb disease, which is characterized by depression, anemia, icterus, and hemoglobinuria clinically. Both *C. perfringens* type B and C strains produce the necrotizing and lethal β-toxin that is responsible for severe enterotoxemia in young lambs, calves, pigs, and foals, showing as acute dysentery with fetid, blood-tinged feces, toxemia, and a high mortality [104]. Colostrum containing trypsin inhibitor increases the susceptibility of young animals to proteolytic enzyme- sensitive β-toxin. Another kind of enterotoxemia, also called pulpy kidney disease or overeating disease is caused by *C. perfringens* type D released epsilon toxin with predisposing factors, such as a carbohydrate-rich feed intake [104]. Young animals are more susceptible to develop into a peracute form due to the lack of relevant immunity. Excitement, convulsions, or even sudden death may follow typical clinical signs, such as abdominal pain, watery or bloody diarrhea, hyperglycemia, or glycosuria [105]. Since epsilon toxin also causes damage to the capillaries of the brain, some neurological clinical signs of opisthotonos and circling are present sometimes [102]. Symptoms are correlatable with the necropsy finding, such as myocardium haemorrhage, abdominal muscles and intestine cicatrix, and kidneys postmortem autolysis.

3. *C. perfringens*-Induced Enteritis, Immunity, and Microbiome

As discussed in the above sections, *C. perfringens* induces a large array of acute diseases in various organs/tissues with rapid disease progression, such as gas gangrene (myonecrosis) and enteritis in humans, swine, cattle, and chickens. Despite the different actions of the toxins, most of them are pore-forming toxins and induce cell death [50], as discussed in Section 1. A successful *C. perfringens* infection is associated with sufficient bacterial inoculum accessing to anaerobic internal organs/tissues through wounds, inflammation, or others [15,106]. Hence, it is certain that *C. perfringens*-induced diseases in animal organs/tissues share common host–pathogen interactions and pathogenicity. Here, we discuss the disease and the interaction between *C. perfringens*, immunity, and the microbiome in myonecrosis and enteritis.

3.1. The Interaction of C. perfringens and Immunity in Myonecrosis

Gas gangrene or myonecrosis is a rapid life-threatening condition with muscle necrosis, gas production, and sepsis, often occurring in traumatic wounds with bacterial contamination and surgery involving the gut [49]. Unlike enteritis of complex interactions between *C. perfringens*, microbiota, and immunity, myonecrosis mainly has interactions between the pathogen and the hosts immunity [107]. It would not be surprising that the underlying mechanisms in myonecrosis are applicable to enteritis. The disease progression of gas gangrene in mice is rapid with signs of limping and swelling of the footpads and thighs, displaying as early as 2 h post-infection, while extensive muscle necrosis at the infected site is seen at 4 h post-infection [108]. Systematically, α-toxin (PLC) induces a dose-dependent reduction in myocardial function in isolated rabbit atrial preparations, while α- and θ-toxins (Perfringolysin O) cause profound hypotension and bradycardia in mice within 40 min, reaching a lethal point [49].

A unique feature of gas gangrene is the lack of phagocytic cells in the infection site, which is one of main factors leading to the uncontrolled infection spreading [106]. Human-circulating leukocytes have about 10% monocytes and 50–70% neutrophils, whereas mice have 4% monocytes and 10–25% neutrophils [109–112]. Monocytes trafficking to peripheral tissues via the bloodstream are comprised of heterogeneous myeloid cells with original progenitors in the bone marrow [113]. Monocyte migration and infiltration are guided by chemokines secreted from cells in their destination [114]. The cell migration process follows a general paradigm of leukocyte adhesion and trafficking, and is mediated by the interactions of a number of adhesion molecules [115]. After infiltration into the destination and conditioning by local growth factors, pro-inflammatory cytokines, and microbial products, monocytes differentiate into macrophage or dendritic cell populations, while most tissue-resident macrophages and classical dendritic cells are maintained independently of monocyte origin [116]. Phagocyte infiltration reduction is in a dose-dependent manner with *C. perfringens* inoculum doses [106]. Phagocyte infiltration is inhibited in the first 3 h post-infection in mice infected with a lethal dose of 1×10^9 CFU *C. perfringens* while the sublethal dose of 1×10^6 CFU bacterium is not. By depleting circulating monocytes or neutrophils before intermediate *C. perfringens* infection, it shows that monocytes inhibit gas gangrene, while neutrophils slow the onset of the disease but are not protective [106], suggesting that monocytes may be more resistant to the medium-dose pathogen sabotage and/or more effective to clear the pathogen from the infection site.

C. perfringens reduces mature neutrophils in bone marrow, peripheral blood, and infected muscle in an α-toxin-dependent manner, while α-toxin does not change the cell viability [117]. θ-toxin is cytotoxic to polymorphonuclear leukocytes and macrophages, but the cytotoxicity does not significantly cause the absence of the phagocytes [117]. At the molecular level, α-toxin upregulating adhesion molecules on the surface of immune cells promotes intravascular cell aggregation and subsequent vascular occlusion, causing neutrophils to adhere and intravasate at locations remote from the infected site [51]. In addition, α-toxin-mediated activation of platelet gpIIbIIIa induces the formation of microvascular thrombosis and occlusions, which blocks the blood flow to the infection site, causes ischemic tissue necrosis [47], and impedes neutrophil diapedesis by aggregating to adherent platelets by platelet P-selectin (CD62-P) [118].

An accumulating body of scientific evidence has suggested that the host innate immune system is enhanced with granulopoiesis to help hosts against bacterial infection. The impaired innate granulopoiesis promotes low-dose *C. perfringens* infection to a life-threatening myonecrosis disease [119]. Granulopoiesis is mediated with cytokine granulocyte colony stimulating factor (G-CSF), which is secreted from various cells responding to interleukin-17A (IL-17A) [120]. IL-17A is produced by Th17, $\gamma\delta$-T cells, and type 3 innate lymphoid cells (ILC3) [121,122]. The release of IL-17A is in turn mediated by cytokine IL-23 secreted from tissue-resident macrophages and dendritic cells [122]. Three types of neutrophil granules are present in mature neutrophils, and these granules are composed of proinflammatory contents [123]. In addition, neutrophils generate a neutrophil extra-

cellular trap (NET), a web-like extracellular structure of DNA covered with histones and enzymes such as neutrophil elastase and myeloperoxidase (MPO). NETs are abundant at inflammatory sites with neutrophils [124]. Few reports are available on the relationship between NETs and gas gangrene, in part, because of neutrophil reduction with the existence of *C. perfringens*.

The nature of rapid disease progression in *C. perfringens*-induced gas gangrene implies that there is not enough time for the host to invoke adaptive immunity. Indeed, few papers have talked about the role of adaptive immunity against the infectious bacteria, except in vaccinations. As discussed in Section 4, the targets of vaccines are mainly *C. perfringens* toxins and enzymes. So far, vaccines against *C. perfringens* virulence factors are the best options to prevent the infectious disease.

3.2. The Interaction of C. perfringens, Immunity, Microbiota, and Its Metabolites in Enteritis

Besides inducing myonecrosis in the infected muscle, *C. perfringens* causes enteritis in humans and animals. The pathogens induces human enteritis necroticans, acute watery diarrhea (food poisoning), non-foodborne diarrhea, preterm infant necrotizing enterocolitis (NEC), and animal enteritis, including equine acute NEC, swine enterocolitis, chicken necrotic enteritis (NE), and enterotoxemia in adult cattle, sheep, and goats [50]. Enteritis is often assisted by predisposing factors. Extensive research in chickens shows that *C. perfringens* induces NE in chickens often predisposed to coccidia *Eimeria maxima* or *E. acervuline* infection [125], high fishmeal [89] or a high fiber diet [126]. The preexisted factors may induce intestinal stress from intestinal inflammation to injury, leading to bacterial overgrowth and a more anaerobic atmosphere, favorable for *C. perfringens* growth. As expected, controlling the predisposing factors greatly reduces NE outbreak. In the poultry industry, reducing *Eimeria* infection effectively decreases NE.

Similar to myonecrosis, the immune response of *C. perfringens*-induced enteritis is important in the pathogenesis. Some stool samples of the NEC patients are detected as *C. perfringens*-positive, and NEC could be related to the pathogen infection [81]. The hyperinflammation in NEC is dependent on NET, because NEC severity is significantly reduced in mice incapable of forming NETs by protein arginine deiminase (PAD) inhibition, and NET is correlated positively with NEC progression [127]. Because no reliable animal model for *C. perfringens*-induced enteritis in human and less frequent enteritis in other animals, the most active investigation of *C. perfringens*-induced enteritis is poultry NE. Chickens challenged with *C. perfringens* alone show increased jejunal gene expression of proinflammatory cytokines *Il1β* and *Il17* (Th17 cytokine) and Th2 cytokine *Il13*, but reduced anti-inflammatory cytokine *Tgfβ* [128]. Interestingly, *C. perfringens* infection does not reduce the bird growth performance of body weight gain and feed efficiency in this report. In a combined challenge trial with dietary wheat, *Eimeria*, and *C. perfringens*, Daneshmand and colleague show that NE with *Eimeria* is the optimal model to reproduce NE [129]. They also find that jejunal gene expression of *Il6*, *Il10*, *Il2*, and annexin-A1 is increased in NE birds. *E. maxima* infection alone reduces host defense peptides avian beta-defensins 1 (*AvBD1*) in the ileum, *AvBD11* in the jejunum and ileum, and *Leap2* in all three small intestinal segments [130]. The mRNA levels of proinflammatory mediators, such as *Infγ*, *Litaf*, *Il1β*, and *Mmp9*, in ileal tissue are increased in *E. maxima*- and *C. perfringens*-double infected chickens, while inhibiting COX signaling by aspirin significantly attenuated INFγ-induced inflammatory response in the splenocytes and ileal tissue of NE birds [9]. The importance of the immune response is shown in the cases of worsened NE in immunocompromised birds challenged with infectious bursa disease (IBD) vaccines [126] or with stress hormone corticosterone [131]. Similar to gas gangrene, few reports are available to elucidate the adaptive immune response in NE and future research is much needed.

Unlike in the myonecrosis of pathogen-host interactions alone, *C. perfringens*-induced enteritis has interactions between the pathogen, the host immunity, and the intestinal microbiota. Because of the proximity, the gut immune system and microbiota are actively interplayed with each other against pathogens and foreign substances [132]. Gut mi-

croorganisms form a microbial community co-existing with the gut-associated lymphoid tissue [133], which is the largest immune organ in animals. Under healthy status, the intestinal epithelium and resident microbiota are separated by a mucus layer, which not only provides static shielding, but also limits normal microbiota immunogenicity by priming dendritic cells [134,135] to differentiate antigens present by normal microbiota and invading pathogens [136]. The normal microbiota has the capacity to induce the lymphoid tissue's immune response to protect the host from pathogen infections [137]. Hence, the normal microbiota can live along with the host without causing harm, or getting removed by the host immunity [138]. The elimination of microbiota results in a deficient function of immunity, as a fact, antibiotic-treated mice or germ-free mice are often used as a model for the study of pathogen colonization [139]. Infectious pathogens often break the balance of the gut microenvironment to induce detrimental effects, which may cause the gastrointestinal illnesses such as *C. perfrigens*-induced enteritis.

The studies on NEC are some of the most actively studied and relevant fields to *C. perfrigens*-induced enteritis, although not all NEC is induced by the pathogen. The relative abundance of *C. perfringens* (8.4%) and *Bacteroides dorei* (0.9%) is increased in the meconium (newborn's first feces) of human neonates developing NEC compared with healthy ones (0.1% and 0.2%, respectively), while in post-meconium samples, the abundance of *Staphylococci* is inversely associated with NEC development, and *C. perfringens* continued to be prevalent in NEC babies [81]. NEC rarely develops before 10 days of life when the microbiota starts to assemble in the newborn gut [140], and NEC is associated with an increased usage of antibiotics, especially prolongation use in the first 7 days of life [141], which disrupts microbiota assembly. NEC is developed in very low birthweight infants with the increased relative abundance of class γ-Proteobacteria and reduced strict anaerobic bacteria (especially of the class *Negativicutes*) [142]. Stool samples from NEC babies at 3 and 7 days show a 34% bloom of the phylum *Proteobacteria* and a 32% decrease in the phylum *Firmicutes* with class γ-Proteobacteria frequently detected in NEC samples [143]. The microbiota of postnatal NEC cases lacks the genus *Propionibacterium* and is preceded with either increased phylum *Firmicutes* during days 4 to 9 or the phylum *Proteobacteria* during days 10 to 16 [144]. Microbiota diversity and the relative abundance of the phylum *Actinobacteria* and class *Clostridia* are significantly lower in stool samples of infants with NEC compared to controls [145]. Together, these reports show the association of the specific members of microbiota with NEC, but further research on the relationship between NEC, *C. perfringens* and microbiota is much needed.

Because of the significant burden of NE in the poultry industry, research on the interaction of microbiota and *C. perfringens* is actively pursued. In a co-infection experiment of *E. maxima* and *C. perfringens*, the relative abundance of *C. perfringens* increased to 58–70% of NE birds compared to 0.02% of healthy chickens with a majority of the ileal microbes, such as members of the phylum *Firmicutes*, being reduced [146]. The number of operational taxonomic units (OTU) is reduced in Cobb but increased in Ross and Hubbard birds compared to the uninfected ones, while the relative abundance of *Lactobacillus* is increased in Cobb but decreased in Hubbard and Ross birds [147]. Interestingly, the relative abundance of phylum *Actinobacteria* is reduced, while the relative abundance of the genera *Butyrivibrio, Lactobacillus, Prevotella,* and *Ruminococcus* is increased in NE Cobb 500 birds [148]. In a *C. perfringens* infection alone experiment, pre-infection microbiotas do not impact NE outcomes, while *C. perfringens* challenge NE bird microbiotas with the reduced class *Clostridia* [149]. In the three co-infection NE experiments with different setups, microbiota diversity and composition are not consistently changed with NE [150], suggesting the complicated relationship between the microbiota and NE.

The microbiota could interact directly with pathogens or the host or indirectly communicate with them through their metabolic activity, such as through metabolites. We have reported that the microbiota metabolite of secondary bile acid deoxycholic acid (DCA) reduces co-infection of NE with reduced inflammatory mediators of *Infγ*, *Litaf*, *Il1β*, and *Mmp9* mRNA accumulation in ileal tissue [9]. DCA reduces the NE-induced ileal

inflammation in a dose-dependent manner compared to NE control birds, while total bile acid and DCA are increased in proportion to dietary DCA levels [151]. The following work shows that DCA and LCA reduce NE histopathology, suggesting that secondary bile acids, but not total bile acid levels, control NE [45].

3.3. Ischemia in C. perfringens-Induced Diseases

Organ or tissue ischemia is a status with occlusion of the arterial blood supply and results in a severe imbalance of metabolic supply, demand, and exchange (hypoperfusion), causing tissue hypoxia [152]. Tissue hypoperfusion of ischemia occurs during sepsis, acute coronary syndrome, organ transplantation, limb injury, and intestinal blood blockage. Interestingly, reperfusion by restoring blood flow and reoxygenation exacerbates tissue injury with a profound inflammatory response, an effect of reperfusion injury [153]. *C. perfringens*-induced necrotic diseases have typical ischemia characteristics, which is often overlooked. Several factors contribute to the ischemia: (1) α-toxin induces vascular occlusion [51]. The rapid destruction of muscle in α-toxin-induced rat gas gangrene involves toxin-mediated irreversible impairment of local and regional blood flow [154]. (2) The rapid gas accumulation generated from *C. perfringens* fast growth increases interstitial pressure at the infected sites [155] and reduces blood flow. The gas pressure in the muscle [155] or intestine [48] also squeezes blood vessels and breaks muscle or intestine integrity. (3) The gases generated by *C. perfringens* are mainly the anaerobic gases CO_2, H_2, H_2S, N_2, and others [156]. The anaerobic gases further contribute to the severity of ischemia. (4) H_2S, a pungent odor of rotten eggs, is a vasodilation gas by opening adenosine triphosphate–sensitive K (ATP) channels in vascular smooth muscle [157] and reduces arterial capillary blood pressure, resulting in edema and the swelling of villi. (5) *C. perfringens* enzymes and its toxins increase capillary permeability, reduce osmotic pressure and net filtration or absorption pressure, resulting in edema [103], the swelling of villi [9], and a reduction of capillary exchange of O_2, nutrients, CO_2, and other wastes, leading to exacerbated ischemia.

The underlying mechanism of ischemia injury is hypoxia that leads to anaerobic cellular respiration and dysfunctional electron transport chains in mitochondria with ATP production reduction [158]. The decreased ATP impairs energy-dependent Na^+/K^+ pumps, sequesters Na^+ inside the cell, and reduces Ca^{2+} excretion causing an intracellular calcium overload that generates reactive oxygen species (ROS) and activates NADPH oxidase [159], resulting in the cellular accumulation of hydrogen, sodium and calcium ions and subsequent hyperosmolarity, water influx, cell swelling and death. A Ca^{2+} blocker, verapamil, attenuates intestinal ischemia and reperfusion injury, presumably through reducing the intracellular Ca^{2+} influx [160]. Intraportal verapamil administration reduces the ischemia–reperfusion injury caused by free oxygen radicals [161].

4. Prevention and Treatment of C. perfringens-Induced Necrotic Diseases

Because *C. perfringens*-induced necrotic diseases are sporadic and acute in humans, it is difficult to predict the risk and to take necessary preventative measurements. The lack of human vaccine development is one of the outcomes. *C. perfringens* infection is anticipated in food animal production, and the cost-effective vaccines are hence actively developed, mainly targeting the pathogenic toxins and enzymes, such as the PLC vaccine. In addition, the treatment of *C. perfringens*-induced necrotic diseases in human is to prevent the further spread of infection, such as antibiotics, wound debridement, and the surgical removal of dead or infected tissues with adjuvant hyperbaric oxygen therapy (Table 2).

Table 2. Prevention and treatment of *C. perfringens*-induced necrotic diseases.

Prevention
∗Vaccine
1. Bacterin vaccines
2. Toxoid vaccines
3. Recombinant proteins vaccines
∗Potential new interventions
1. Monoclonal antibodies (mAb)
2. Blood thinners (anticoagulants)
3. Hypoxia relief
4. Cell death inhibitors
5. Probiotics, gut microbiota and its metabolites
Treatment
C. perfringens-induced myonecrosis and severe enteritis
1. A combination of intravenous high doses broad-spectrum antibiotics
2. Wound debridement of all bacteria carriers
3. Surgical removal of dead or infected tissues
4. Hyperbaric oxygen therapy

4.1. Preventing C. perfringens-Induced Diseases with Vaccines

Because *C. perfringens* frequently infects foodborne animals, the prevention, particularly vaccines, is actively developed. Vaccines are the most effective way to reduce infectious diseases, their complications, and their spreading in animals by generating protective antibodies and other immune responses [162]. Many types of vaccines have been developed to prevent *C. perfringens*-induced diseases, such as bacterin, toxoid, and recombinant vaccines. Similar to the discussion in Section 2.2, two main NE chicken models are used to assess *C. perfringens* vaccines and the result needs to be carefully interpreted based on the context.

Bacterin vaccines refer to a suspension of killed or attenuated bacteria for use as a vaccine. For *C. perfringens*, the vaccine is produced by inactivating the *C. perfringens* cells with formaldehyde [163]. The bacterin vaccine induces a comprehensive immune response against all the infection-associated surface antigens, but it also stimulates the unnecessary antibodies against non-important epitopes [164]. Unlike other *Clostridia* pathogens, the bacterin vaccine fails to protect NE birds in a NetB positive *C. perfringens* infection alone study [165].

Instead of targeting the whole bacteria itself, toxoid vaccines are used to create immunity against the disease-relating toxins. Formaldehyde-inactivated crude toxoid from the supernatant of *C. perfringens* type A and/or C is vaccinated in breeder hens to prevent subclinical NE in their progeny broiler chickens in a natural infection model [166]. A beta toxin-based toxoid vaccine was developed to control type C-induced toxemia in the mouse intravenous injection model [167]. NetB-positive strains are frequently associated with field NE incidence, and formaldehyde-inactivated NetB toxoid or NetB genetic toxoid (W262A) were developed and can induce antibody responses against NetB and partially protect against NE in the *C. perfringens* infection alone model [5]. The use of the alpha toxin isoform (αAV1b) with a natural mutation at residue 11 (His/Tyr) has also been considered a promising alternative, which is able to induce an immune response and with an anti-alpha toxin titer (24.0 IU/mL) in rabbits [168]. Besides toxins, lipoproteins on the *C. perfringens* surface have potential virulence. Purified *C. perfringens* lipoproteins stimulated protective immunity in a mouse model of gas gangrene [169].

Another vaccine development option is to use recombinant proteins because the toxin sequences are well known, and the protein expression system is well established. CPA is the main toxin responsible for *C. perfringens*-induced gas gangrene. CPA has two domains, one is the amino-terminal domain with a phospholipase C active site and zinc ions, and another is the carboxyterminal domain with a phospholipid binding ability.

The recombinant vaccine using non-toxic C-terminal (Cpa247–370) domains of the CPA neutralized both phospholipase C and hemolytic activities and it protected mice against experimental gas gangrene with more than 10x LD100 doses of *C. perfringens* type A infection [30]. Another recombinant vaccine with C-terminal (Cpa247–370) domains and glutathione-S-transferase (GST) was expressed on the surface of Bacillus subtilis PY79 spores and it protected mice immunized nasally or orally against 12× LD50 of alpha toxin challenge [170]. A recombinant vaccine with pT1NX-alpha expressed on the surface of *Lactobacillus casei* ATCC 393 elicits mucosal and humoral immune responses and protects against 900× LD50 alpha-toxin in BALB/c mice [171]. The *Lactobacillus* system was also used for expressing ETX to protect mice against enterotoxemia [172]. A bivalent chimeric protein r-Cpae vaccine using both C-terminal binding subunits of CPA and CPE generated high systemic IgG, intestinal mucosal s-IgA, and increased the survival rate after toxin challenge [173]. A purified recombinant CPF_2918 vaccine induced antibody production, a Th1 and Th2 response, and partially protected mice in direct *C. perfringens* challenge [174].

4.2. Treating C. perfringens-Induced Diseases

C. perfringens-induced myonecrosis and severe enteritis is a rapid-progressive and highly lethal infectious disease; thus, the urgent diagnosis and treatment of patients at high risk is crucial. After diagnosis of myonecrosis, treatment usually involves using a combination of intravenous high doses of broad-spectrum antibiotics, wound debridement of all bacteria carriers, and surgical removal of dead or infected tissues with adjuvant hyperbaric oxygen therapy [175]. The antibiotics include growth inhibition of penicillin or cephalosporin, exotoxin inhibition of clindamycin or tetracycline, and others such as aminoglycoside, cephalosporin, vancomycin, linezolid, and metronidazole [176]. Because most antibiotics are hydrophilic and are unable to penetrate ischemic muscles sufficiently to be effective, they are only used as an adjuvant treatment. Unfortunately, in most cases, to relieve compartment pressures by fasciotomy, the final surgical removal of infectious areas in a hyperbaric chamber are necessary, because the oxygen-rich atmosphere prevents the further growth of *C. perfringens* and its toxins produced in the infected tissues [40,177]. The surgery restraints further tissue ischemia and necrosis and induces neovascularization and tissue repair with capillary budding by increasing the production of vascular epidermal growth factor (VEGF) [40,178]. Similarly, after a diagnosis of severe enteritis such as NEC in preterm neonates, surgery between primary peritoneal drainage or laparotomy is recommended to control sepsis and remove any gangrenous bowel preserving as much bowel length as possible [179].

4.3. Potential New Interventions against C. perfringens-Induced Necrotic Diseases

As discussed in the above sections, it is highly anticipated that new therapies will emerge based on new disease mechanism insights and technology developments, such as monoclonal antibodies (mAb), anticoagulants, hypoxia relief, and microbial metabolites. mAbs are laboratory-made proteins that mimic the immune system's ability to fight off harmful pathogens, which have attracted much attention during the treatment of SARS-CoV-2 in recent years. Thousands of mAbs have been developed as drugs since 1985 [180] and there are increasing studies focusing on mAbs against bacterial and viral infectious diseases such as *C. perfringens*-induced diseases. In 2014, Garcia and his colleagues expressed the variable region sequences of ETX (4D7) by *Nicotiana benthamiana*, and the purified mAbs (ETX mAb c4D7) exhibited highly prophylactical and therapeutic effects for ETX intoxication in mice [181]. In 2018, rabbit ETX oligomerization-blocking mAbs produced by B-cell immuno-panning and cloning techniques were evaluated in their ability to inhibit ETX endocytosis and cellular vacuolation in vivo, as well as to protect against ETX-induced death in C57BL/6J mice [182]. Recently, our group has been working on combining gene cloning and Phage Display technologies to generate mAbs against *C. perfringens* for the treatment of its induction of necrotic enteritis.

Localized blood clotting following ischemia is a common sign of gangrene, which may assist the proliferation of anaerobic *C. perfringens* and its toxins production, which eventually develops into rapid tissue destruction and entire limb destruction. The interactions between toxins and the host's coagulation system contribute to the pathogenesis of the gas gangrene [183]. During the treatment of *C. perfringens*-induced gas gangrene, medicines such as blood thinners (anticoagulants) might be used to prevent blood clot augmentation and to restore the high-level oxygen environment. The calcium channel blockade that is currently used to treat acute myocardial infarction, with its ability to prevent vascular occlusion and maintain tissue viability, may be a choice for the treatment of the gas gangrene [183].

One of the gas gangrene-related symptoms, hypoxia, refers to a condition in which oxygen is limited and is always associated with poor clinical outcome. It has been demonstrated that Hypoxia-inducible factor (HIF) plays an important role in the hypoxia signaling pathway [184], such as the induction of a single gene erythropoietin, and the upregulation of one hundred downstream targets. Accordingly, HIF and its downstream targets are emerging as novel therapeutic options to treat various organ injuries. It is anticipated that agents reducing HIF may be used to treat *C. perfringens*-induced diseases.

C. perfringens uses its large arsenal of toxins to produce intramuscular and intestinal infections in humans and livestock. This host–toxin interaction relies on specific receptors on the plasma membrane of target cells, and may result in the activation of cell death pathways, such as apoptosis, necrosis and/or necroptosis [3]. So, instead of targeting the toxins, treatment focused on blocking cell death signaling pathways is a viable option. The pharmacological COX signaling inhibitor aspirin, attenuated a *C. perfringens*-induced ileal inflammatory response, intestinal cell apoptosis, and BW loss in NE birds [9]. CPE-induced apoptosis (low dose) and necrosis (high dose) induce receptor-interacting serine/threonine-protein kinases 1 and 3 (RIP1 and RIP3) and mixed-lineage kinase domain-like pseudokinase (MLKL) oligomerization in human enterocyte-like Caco-2 cells, while the activation of calpain is involved in CPE-induced necroptosis [185]. Thus, potential therapeutics, such as inhibitors of RIP1, RIP3, MLKL oligomerization, or calpain, may prevent and treat CPE-mediated diseases.

C. perfringens overgrowth and enteric infection is part of the disorder of the gut environment. A variety of probiotics, such as *Bacillus*, *Lactobacilli*, *Enterococci*, and *Bifidobacteria* [186], have been used as prophylactic feed supplements for NE control. They compete with the growth of *C. perfringens* in the intestine [187] as well as enhance mucosal immunity, degrade apoptosis-leading proteins, and modulate the expression of inflammatory cytokines [186]. For example, *Lactobacillus plantarum* 16 (Lac16) and *Paenibacillus polymyxa* 10 (BSC10) in feed (1×10^8 cfu·kg^{-1}) can resist against *C. perfringens* type A strain infection in broilers, showed the reduced expression of the apoptosis-related gene Bax, p53, less intestinal damage, stabilized intestinal microbiota balance and microbiota metabolites, B-vitamin, peptidoglycan and pyruvate biosynthesis [188]. Dietary *Bacillus licheniformis* H2 isolated from resistant chicken ileum enhances the body weight gain, serum antioxidant index, and the expression of lipid-metabolism genes in NE chickens [189].

The normal gastrointestinal tract involves a certain composition of microbiota and its derived metabolites, such as bile acids, short-chain fatty acids, branched-chain amino acids, trimethylamine N-oxide, tryptophan, and indole derivatives [190], some of them play important roles in the restriction of pathogenic bacteria and help maintain the gut homeostasis. One of the microbial metabolic by-products, secondary bile acid deoxycholic acid (DCA), inhibits 82.8% of *C. perfringens* in vitro growth at 50 μM, and 1.5 g/kg dietary DCA reduces intestinal inflammation and body weight (BW) loss in broiler NE chickens challenged with *E. maxima* and *C. perfringens* [9]. Further cellular and molecular analysis confirmed that DCA prevents NE-induced BW loss and ileal inflammation through attenuating the inflammatory response, and DCA has the potential to be an antimicrobial alternative against NE. Some natural components also show a similar effect. In Carrasco's work [191], he reviewed two kinds of natural plant extracts, tannins and essential oils,

as alternative feed additives against *C. perfringens* by their in vitro antibacterial and anti-toxin activities. As a natural component in A. membranaceus, astragalus polysaccharide (APS) was investigated by Song [192] as a dietary supplementation to alleviate intestinal inflammation in broilers challenged with NE through regulating intestinal microbiota and metabolite diversity that related to inflammation Th17/Treg balance.

5. Conclusions

Given the fast research advancement in immunology, microbiota, and metabolomics, it is a better time than ever to investigate the mechanisms of the interactions between *C. perfringens*, immunity, and microbiota, and to use the knowledge to develop new interventions against the pathogen-induced myonecrosis and enteritis. *C. perfringens* expresses a large array of toxins and enzymes detrimental to humans and animals. *C. perfringens*-induced myonecrosis and enteritis is the combination of toxin, toxin-induced ischemia, toxin-induced phagocyte absence, blood occlusion, and a necrosis-induced inflammation storm. The best option to prevent *C. perfringens*-induced necrotic diseases is to immunize animals with vaccines that mainly target the pathogen's toxins and virulence enzymes. Treatments include the surgical removal of infected and necrotic tissue, antibiotics for blocking further pathogen dissemination, microbiota metabolites to reduce *C. perfringens* growth, and anti-coagulation drugs to reduce blood occlusion. Additional research is greatly needed to open new avenues to elucidate the in-depth understanding of the role of immunity and microbiota and to develop new therapeutic approaches to control the myonecrosis and enteritis. Together, it is essential to understand the disease etiology, immunity, and microbiome for specifically targeting the pathogenic contributing factors to prevent and treat the necrotic diseases.

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