A short overview of *Clostridium perfringens*: Relevancy, Toxinotypes, Clinical Impacts, and the Challenges of Biofilm Formation

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Abstract
C. perfringens, a ubiquitous bacterium, is implicated in a series of recent outbreaks across Europe, reflecting its significance in public health. The organism manifests different clinical presentations in both human and animal hosts. In the realm of veterinary science, its pathogenesis in species such as poultry, cattle, swine, and equines is of particular concern due to the economic implications stemming from morbidity and mortality rates. The enterotoxins produced by this bacterium is the primary cause of symptoms like watery diarrhea, abdominal pains, and cramps in humans, and leads to conditions such as clostridial myonecrosis and necrotic enteritis in animals. An important aspect of its virulence strategy is its ability to form biofilms. These sophisticated microbial assemblages confer an augmented resistance against environmental challenges and antimicrobial interventions. This review consolidates information about *C. perfringens* outbreaks in Europe, mode of infection, pathogenesis in various animals, associated clinical symptoms, and the nuances of its biofilm formation. The present literature synthesis could provide valuable insights for forthcoming preventive and therapeutic initiatives.

Keywords: *Clostridium perfringens*, infection and pathogenesis, toxinotypes, clinical manifestations, biofilm formation

1. Introduction

C. perfringens, a Gram-positive bacterium, thrives in diverse environments like soil, water, and even the gastrointestinal tracts of humans and animals. Initially discovered at Johns Hopkins Hospital in 1891 and named *Bacillus welchii* [1], its significance in human and veterinary medicine is largely attributed to its ability to produce an arsenal of about 20 virulent toxins [2]. These toxins associate with a spectrum of ailments, from benign gastrointestinal disturbances to severe conditions like gas gangrene and gastroenteritis in both humans and animals [3] [4]. The economic implications of its pathogenicity are felt particularly in the animal feed industry, as it incurs considerable losses through afflictions like severe enteritis in poultry and pigs [5].

The pathogenesis of *C. perfringens* infections is multifaceted and closely related to the toxins it produces. Different 7 toxinotypes (A, B, C, D, E, F and G) of the pathogen have been delineated based on their toxin profiles, each correlate with specific diseases which are represented in Figure 1 [6]. Furthermore, the mechanism of infection is not solely dependent on the toxins such as alfa (α -CPA), beta (β -CPB), epsilon (ε -ETX), iota (ι -ITX), *C. perfringens* enterotoxin (CPE) and

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necrotic enteritis B-like toxin (*NetB*) toxins [7]. Among the seven identified subtypes, a salient example is the Type A strain, which is notorious for causing gas gangrene, a condition leading to complications such as muscle necrosis, systemic shock, and even mortality in extreme cases [8, 9].

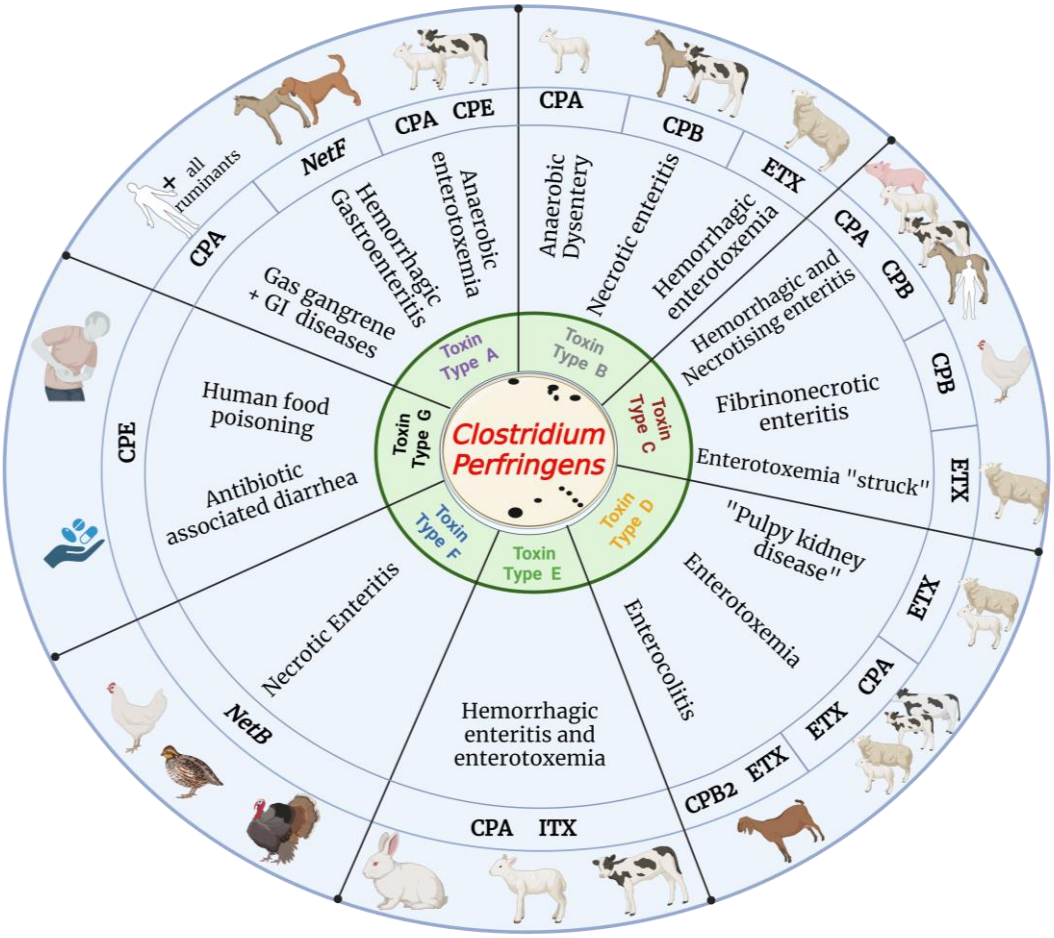

Figure 1. Diseases associated with *C. perfringens* toxinotypes and with the produced toxins in humans and animals. Created via Biorender.com.

The accelerated progression of infections due to *C. perfringens* has occasionally resulted in patient demise even before a definitive diagnosis, indicating a potential capability of this pathogen to circumvent the host's primary immune defences.

The bacterium's ability to rapidly proliferate under anaerobic conditions, in tandem with its endospore's resilience, ensures its survival in diverse environments, facilitating recurring infections. Interestingly that the pathogen was also identified in honey, particularly strain A, which has the ability to produce the α-toxin [10]. This is noteworthy given honey's well-known antibacterial properties [11]. The spores of this bacterium can survive and thrive for extended periods in the environment and in the absence of nutrients [12]. Additionally, the bacterium is characterized by its rapid growth in food and vegetative cells, with its population capable of doubling in less than 10 minutes [13]. However, due to the heterogeneity in germination, some spores germinate within minutes, while others can take hours or even longer sporulation time. Understanding the transmission modes of pathogens is pivotal when devising control strategies against foodborne diseases; however, other factors must also be considered [14]. Meat, especially beef and poultry, as well as meat-containing products, are significant vectors for foodborne illness caused by *C. perfringens*. During the processing of carcasses and meat in slaughterhouses, contamination from interactions

with contaminated industrial surfaces serves as a pathway for pathogen transmission and contributes to the emergence of biofilms in the food production sector [15]. Nevertheless, this pathogen can also be found on plant-based products, including spices and herbs, both in raw and processed forms. Notably, the spores of certain *C. perfringens* strains can endure even in boiling water for up to an hour [16].

Antibiotic resistance is an escalating concern in the realm of microbial pathogens, and *C. perfringens* is no exception. Recent years have witnessed an uptick in reports documenting strains of this bacterium exhibiting resistance to traditionally used antibiotics, complicating treatment protocols [17]. A recent study from Romania has reported type A and cpa-positive *C. perfringens* strains isolated from the pig faecal samples with a potent resistance to tetracycline, penicillin, enrofloxacin and erythromycin [18]. These resistance traits, often conferred by mobile genetic elements such as plasmids, highlight the adaptive prowess of this pathogen in the face of therapeutic challenges [17]. Adding to its intricate nature is its capability to form biofilms – complex bacterial communities shielded within an extracellular matrix (EPS) adds another layer to its pathogenicity [19]. This not only offers protection against external threats but also boosts resistance to antibiotics and immune responses. Unravelling the intricacies of bacterial cell structures and signaling pathways involved in this process is pivotal for devising strategies to prevent or disrupt these protective bacterial communities [20].

This review briefly reflects on *C. perfringens'* pathogenesis, delving into its modes of infection, the challenges posed by its toxinotypes, clinical manifestations, and the role of bacterial cell structures in biofilm formation, aiming to shed light on potential therapeutic interventions and preventive measures.

2. Recent *C. perfringens* outbreaks data

The globalization of food supply, production, and distribution, coupled with the emergence of new pathogens and an increasing number of consumers choosing to dine out rather than cook at home, amplifies the risk of foodborne disease outbreaks. *C. perfringens* ranks as the second leading cause of bacterial foodborne illnesses in the United States (with ≈ 1 million cases annually) and the United Kingdom. In Europe, it stands as the 4th most common cause [21]. These outbreaks are challenging to control. *C. perfringens*, a ubiquitous bacterium, was identified as the fourth leading cause of foodborne outbreaks in Europe in 2019 [22]. The majority of outbreaks were attributed to *C. perfringens* strains that produce the enterotoxin CPE, encoded by the *cpe* gene [22]. The CPE enterotoxin forms active pores in cellular membranes, leading to an increased influx of calcium and subsequent cell death. This cellular demise results in intestinal lesions, causing fluid and electrolyte loss that can lead to dehydration. Currently, the CPE enterotoxin is under investigation for translational applications, including potential roles in cancer therapy or diagnostics, as well as drug delivery and vaccine administration [23].

From the recent epidemiological studies, 36 food and waterborne pathogens were reported in Europe [24]. The most significant among these were *Salmonella*, *Campylobacter*, *Bacillus cereus*, and notably, *C. perfringens*. Likewise, another study from the Netherlands indicated that the highest incidences were estimated for norovirus, rotavirus, and bacterial toxins produced by *Staphylococcus aureus* and *C. perfringens*. In 2019, 75 water and foodborne outbreaks caused by *C. perfringens* were identified in EU, accounting for 2426 cases of food poisoning in humans [24]. Of these, 27 individuals were hospitalized, and three deaths were reported. The primary sources of contamination were identified as meat, milk, and their derivatives. However, in 2020, the figures reduced to 32 outbreaks and 682 cases of food poisoning, with ten hospitalizations and two reported deaths [24]. It remains unclear whether this reduction was due to the implementation of effective prevention strategies or the 2020 pandemic, which led to population quarantine and a decrease in dining out at restaurants.

3. Toxin classification

C. perfringens is recognized as one of the most ubiquitous bacterial pathogens in the environment and is frequently found in an assortment of foods. It may also be a standard microbial constituent of the intestinal microbiota [10]. While *C.*

perfringens is an inherent component of the normal gut flora, under certain circumstances (e. g., abrupt dietary shifts, antibiotic treatments, or parasitic infections), it can become pathogenic. The pathogenicity of this organism primarily lies in its ability to produce a range of potent toxins and enzymes. *C. perfringens* synthesizes various toxins, encoded by both chromosomal and plasmid genes [25]. The toxins produced by *C. perfringens* exhibit diverse mechanisms of action. Specifically, α-toxin operates through phospholipase, while the binary ι-toxin functions via ADP ribosylation [26]. Conversely, toxins such as CPB, *NetB*, ETX, and CPE mediates pores and facilitate the infection [26]. Research shows that toxins from type A and type F, and occasionally type C, affect humans [16]. α-toxin, encoded by the *cpa* and *plc* gene, is universally produced across all toxigenic types of *C. perfringens*. Perfringolysin (PFO) acts synergistically with α-toxin, leading to progressive tissue damage. During foodborne illnesses, CPE synthesis occurs in the intestines as *C. perfringens* undergo sporulation. Concurrently, sialidases are secrete and improve the pathogen colonization of the intestinal tract and amplify the cytotoxicity of *C. perfringens* [27]. The majority of strains involved in food poisoning have the CPE gene located chromosomally, though occasionally on a plasmid. All examined chromosomal *cpe* (c-cpe) strains produce either *NanH* sialidase or both *NanJ* and *NanH* sialidases which are additional virulence factors [27].

4. Occurrence of the infection

When describing the pathogenesis of *C. perfringens* in animal hosts from a gastroenterological perspective, one should consider the several points. Of which firstly, the pathogenic potential of *C. perfringens* is largely attributed to its production of toxins, whereas each type is associated with different diseases in animals, with Type A being the most common and linked foodborne illnesses, necrotic enteritis in poultry and gas gangrene in mammals [7, 8]. Secondly, upon ingestion of vegetative cells, the bacterium begins sporulation when encountering the stomach's acidic pH. In the context of foodborne illnesses, CPE synthesis happens in the intestines as *C. perfringens* sporulates. Concurrently, sialidases are secreted, enhancing pathogen colonization and increasing cytotoxicity [27]. The vegetative cells adhere to the epithelial lining of the intestines, which is a critical step for the establishment of an infection. Once colonized, the toxins produced by *C. perfringens* cause toxin-mediated damage to the host's intestinal lining. For example, the alpha-toxin, a phospholipase, can cause cell lysis and tissue necrosis. In poultry, the resulting necrotic lesions in the intestines are characteristic of necrotic enteritis [28]. Clinical symptoms vary based on the toxins produced. For instance, ε toxin leads to neurological signs in ruminants due to its effects on the CNS. In neonatal piglets, *C. perfringens* type C causes necrotizing enteritis leading to sudden death. The host's immune system often responds to the infection by producing antibodies against the toxins. However, the rapid progression of the disease can sometimes outpace the host's ability to mount an effective immune response [8]. This highlights the importance of vaccinations, especially in the livestock industry, to protect animals against potential outbreaks.

5. Implications of different toxinotypes in different Animal Species

Each type of toxin is associated with different diseases in animals. *C. perfringens* is an exclusively extracellular pathogen that secretes various cytotoxins instrumental in cell lysis and the breakdown of connective tissues [29]. In animals, particularly in pigs and poultry, it is the causative agent of gangrenous dermatitis, enterotoxemia, and necrotic enteritis [30, 31]. While *C. perfringens* is an inherent component of the normal gut flora, it can be involved in certain diseases across a wide range of both domestic and wild animals, encompassing horses, poultry, birds, rabbits, sheep, goats, cattle, minks, ostriches, dogs, and cats [16].

Type A has the potential to induce diarrhea and enterotoxemia in cattle, sheep, and goats [32]. This manifestation is typified by sudden mortality accompanied by necro-hemorrhagic lesions within the small intestine. Type B, synthesizing β and ε toxins, is implicated in enteritis and enterotoxaemia in various animal species [27]. In contrast, Type C generates only the β toxin, Type D exclusively produces ε toxin, Type E

manufactures the ι toxin, while Type G is responsible for *NetB* synthesis [33]. Each toxin type has been associated with specific disease manifestations. For instance, Type A strains have been linked to enterocolitis in pigs and horses and necrotic enteritis in poultry; they also are accountable for gas gangrene in humans. Types A and C predominantly impact dogs, pigs, and goats [27], specifically the type A strain induces enteric infections in various hosts and is also implicated in histotoxic infections, wherein the α-toxin is deemed the pivotal virulence factor.

Type B strains are notably involved in necrotizing enteritis in sheep and cattle. It's documented that Type C strains induce necrohemorrhagic enteritis in piglets, foals, calves, and sheep [27]. In piglets[34], the etiological progression is characterized by the swift proliferation of *C. perfringens* type C within the jejunum and ileum, compounded by the presence of trypsin inhibitors in the maternal colostrum which hinder the decomposition of beta-toxin (CPB) . Notably, initial perturbations to the epithelial barrier of the small intestine are evident. Furthermore, CPB predominantly targets vascular endothelial cells situated in the mucosal layer and possesses the potential to impede platelet activity [34].

Type D, primarily associated with enterotoxaemia in goats, sheep, and cattle, is a potent clostridial toxin. *C. perfringens* type D is associated with diseases such as enterotoxaemia in goats, sheep, and cattle. Diseases attributed to this type are among the most common clostridial diseases in sheep and goats, sometimes referred to as "pulpy kidney disease" marked by sudden death or respiratory and neurological symptoms [27, 35]. In addition, ε toxin (ETX) produced by *C. perfringens* type D is responsible for inducing acute, often lethal, neurological disorders in ruminant livestock [36].

Type E strains produce a binary clostridial toxin encoded by two plasmid genes, leading to hemorrhagic enteritis and abrupt mortality in animals, notably in newborn calves and type G is primarily related with necrotizing enteritis in poultry [27, 37]. Isolates of Type E are associated with enteritis or enterotoxaemia in various species including rabbits, ruminants (e.g., lambs, bovines, and caprines), and canines [38]. Post-mortem examinations in ruminants typically reveal heightened blood flow (hyperemia) and fluid accumulation (edema) in the intestinal and abomasal mucosa, accompanied by areas of bleeding, acute inflammatory responses, and swelling beneath the mucosal layer [38]. The understanding of the pathogenic mechanisms of Type E remains limited. However, it is widely acknowledged that the ι toxin (ITX) plays a essential role in the virulence of *C. perfringens* Type E strains.

Type F strains of *C. perfringens* are predominantly implicated in foodborne illnesses in both humans and animals [18]. These strains produce CPE, which belongs to the aerolysin family of pore-forming toxins [27]. This enterotoxin is particularly associated with human food poisoning incidents, antibiotic-associated diarrhea, and sporadic non-foodborne illnesses. Identification of the toxin type of different *C. perfringens* isolates is important for an appropriate risk assessment associated with such strains. The CPE is produced during sporulation, binding to the claudin receptor of the tight junctions [18]. Subsequently, it forms a protein complex with other membrane proteins, creating a pore in the membrane. This modification in membrane permeability facilitates the influx of Ca^{2+} ions, along with the loss of Na^{+} and Cl^{-} ions, thereby promoting diarrhea [39].

Contrastingly, the *NetB* toxin-producing Type G is responsible for necrotic enteritis in poultry [40]. Also, enterotoxigenic strains of *C. perfringens* Type F have been implicated in various non-foodborne gastrointestinal diseases, with numerous reported cases of antibiotic-associated diarrhea, sporadic diarrhea, and nosocomial-origin diarrhea [26]. The disease rapidly spreads to healthy tissues, including affecting the kidneys and liver, culminating in shock and often fatality [7, 41]. Studies utilizing animal models suggest that this enterotoxemia involves organ damage, particularly to the heart and kidneys, leading to hyperkalemia, which likely triggers cardiac arrest [42, 43]. Recent studies have identified new *netF*-positive strains and were suggested as future candidates for toxin typing [44].

6. From foodborne illnesses to diverse clinical manifestations

Foodborne illnesses are of significant concern, especially when their source can be traced back to our daily consumables. For instance, foodborne

illness attributed to the CPE toxin which can originate from spores present in the small intestine, commonly found in meats like poultry [16]. This highlights the importance of proper food storage, as improperly stored cooked foods can also serve as a reservoir for these spores. When consumed, the toxin instigated by *C. perfringens*' CPE induces a range of symptoms. Foremost among these are watery diarrhea and severe abdominal pains. Accompanying these may be cramps and nausea. Though the symptoms are intense, they generally resolve within a day. However, it's worth noting that some milder symptoms can persist for up to two weeks [16]. Clinical manifestations associated with CPE comprise abdominal cramping and watery diarrhea without the accompaniment of fever or vomiting, a consequence of pore formation by the toxin. What makes these symptoms particularly concerning is their origin: the CPE toxin modifies the plasma membrane's permeability by increasing calcium influx, leading to cellular death [26]. To put it simply, low doses of CPE create a limited number of pores, allowing a modest influx of calcium. This cellular interference by the toxin leads to observable histological damage, including pronounced villi shortening and intestinal epithelial necrosis [23, 43].

Shifting focus from the intestinal effects, another significant manifestation of C. perfringens is clostridial myonecrosis. This condition is marked by muscle tissue degradation, a direct result of the α and β exotoxins produced by the bacteria [45]. Clinically, this presents as intense pain, swelling, and tenderness in the affected area. Over time, gas production can be observed within the wound. More worryingly, the disease can escalate to cause systemic symptoms. These include shock, renal failure, and arterial hypotension, among others, which can lead to coma and even death [46].

Yet another manifestation of *C. perfringens* to consider is Necrotic Enteritis. This condition is sporadic but particularly affects young animals, causing ischemic, hemorrhagic, or inflammatory necrosis of the jejunum. Specifically, in birds, such as poultry, and other species including piglets, the disease is both severe and potentially fatal [34]. It's primarily caused by *C. perfringens* type A and clinically presents with reduced growth performance, lowered feed efficiency, depression, anorexia, high morbidity, and elevated mortality rates in birds regardless of age. This disease can lead to substantial economic losses, exceeding 6 billion dollars annually due to the high mortality rates in the global poultry sector [47]. Turning our attention to equines, horses are not spared. They suffer from clostridial enterocolitis, which can range from mild diarrhea to severe hemorrhagic conditions, often proving fatal, especially in adult horses and foals [15].

7. *C. perfringens* and biofilm formation

In nature, bacteria predominantly organize themselves into biofilms. Biofilms are typically integrated within an autonomously generated extracellular polymeric matrix (EPS), endowing them with heightened resilience to environmental stressors [20]. More specifically, densely populated pathogenic biofilms like those of *C. perfringens* can become particularly formidable, presenting augmented resilience against antimicrobial agents and the host's immune defenses. The biofilm formation process is organized in three distinct phases: attachment, maturation, and dispersion. Each stage is regulated by a cascade of genetic and environmental characteristics, many of which are only beginning to be fully understood. For instance, the surface proteins of *C. perfringens*, often referred to as adhesins, facilitate the initial binding to surfaces, whether they be biotic (like tissue) or abiotic (like medical implants) [48]. Recent research indicates that *C. Perfringens* possesses a surface protein analogous to the Type IV Pilus observed in *Neisseria* [49]. This protein, comprised of noncovalently bound protein subunits termed pilins, can be dynamically extended or retracted from bacterial cells. Such features facilitate mechanisms like bacterial twitching movement, adherence to host cells, and microbial interactions critical for biofilm development or microcolony establishment [49]. Following attachment, the maturation phase sets in, during which the biofilm gains specific characteristics. Notable among these are enhanced antibiotic resistance, increased resilience against physical stress, and a heightened defense against immune-mediated cells [5]. Finally, the dispersion phase ensures the spread and colonization of new environments. Biofilms aim to shield bacterial cells from exposure to high concentrations of antimicrobials, such as biocides and antibiotics, or even ambient stressors such as

pH, temperature and oxygen [20]. In certain instances, exposure to minimal antimicrobial doses has reduced biofilm formation. However, previous studies indicated that specific isolates increased *C. perfringens* biofilm production when exposed to low doses of tylosin, bacitracin, virginiamycin, and monensin [5]. Conversely, minimal doses of penicillin, lincomycin, salinomycin, and narasin resulted in diminished biofilm formation rates. The majority isolates, both human and animal-derived, of *C. perfringens* were found capable of forming biofilms [5]. Notably, porcine clinical isolates formed more biofilms and showed higher survivability traits than porcine commensal isolates. Sporulation appears to be essential for the production of enterotoxins. It seems that this process occurs even during biofilm formation and persistence, despite most cells within the biofilms being in a vegetative state [50]. Additionally, the ability of extracellular DNA to chelate Mg^{2+} has also been observed in biofilm formation of *C. perfringens* [5].

The underlying mechanisms governing biofilm development involve various genetic and environmental factors. Although it's well-known that the pathogen is aerotolerant, able to survive in soil or water, findings from testing *C. perfringens'* tolerance to atmospheric oxygen have suggested that the biofilm might shield cells from the stress induced by oxygen exposure [5]. This observation has also been seen in other anaerobic bacteria like *Fusobacterium nucleatum [5]*. In oxygen tolerance tests, the average viability rates of *C. perfringens* planktonic cells after being exposed to oxygen for 6 and 24 hours were 63% and 7.4%, respectively. However, when the *C. perfringens* cells were in a biofilm structure, their viability was higher, registering at 80.6% after 6 hours and 61% after 24 hours [5].

Among the genetic pathways influencing biofilm formation, the luxS-regulated gene stands out for its role in biofilm maturation and structural integrity [5]. Meanwhile, environmental factors, like iron availability, can profoundly shape the propensity of *C. perfringens* to form biofilms. In iron-scarce conditions, for instance, the bacterium appears to boost its biofilm production, likely as a survival tactic [51].

In the context of disease and clinical implications, understanding the biofilm life cycle provides valuable insights into devising strategies to counteract *C. perfringens* infections. Novel therapeutics that target specific stages, such as inhibiting initial adhesion or disrupting the EPM, could be pivotal in preventing biofilm formation or eradicating established biofilms. Furthermore, with antibiotic resistance on the rise, biofilm-associated infections are especially challenging. Elucidating the biofilm mode of growth might guide the development of combination therapies that target both the metabolically active and dormant persister cells, offering a multifaceted approach to combatting these robust microbial communities.

6. Conclusions

C. perfringens is a prominent pathogen linked to recent European outbreaks, posing clinical threats to both humans and animals, notably poultry, livestock animals and horses. This bacterium's virulence is emphasized by its toxin-induced symptoms, ranging from human gas gangrene, diarrhea to necrotic enteritis in animals, with significant economic implications, particularly in the poultry sector. Its ability to form robust biofilms complicates infection management due to increased therapeutic resistance. Understanding its pathogenesis and biofilm dynamics is vital for innovating containment strategies. Future studies should emphasize deriving solutions for both isolated and widespread infections.

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