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Optimization of Clostridium septicum Antigen Production and Evaluation of Vaccine Administration Parameters for a Candidate Bacterin-Toxoid to Prevent Dermatitis in Commercial Turkeys

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Optimization of *Clostridium septicum* Antigen Production and Evaluation of Vaccine Administration Parameters for a Candidate Bacterin-Toxoid to Prevent Dermatitis in Commercial Turkeys

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Abstract

The objective of this thesis was to optimize the hemolytic activity of two isolates of *Clostridium septicum* to evaluate if hemolytic titer of *C. septicum* antigen at time of formalin inactivation corresponded to increased serum antibody titer to the *C. septicum* alpha-toxin of turkeys immunized with an experimental formalin inactivated bacterin-toxoid vaccine. Chapter 1 provides a brief overview of the thesis. Chapter 2 consists of a literature review focused on clostridial dermatitis, specifically *C. septicum*-associated dermatitis (cellulitis) that affects commercial turkeys. Chapter 3 outlines our attempts to optimize an experimental autogenous bacterin-toxoid vaccine as a potential strategy to mitigate clostridial dermatitis in commercial turkey flocks.

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Chapter I. Thesis Introduction

Pathogenic clostridial species can cause disease with high morbidity and mortality in both humans and commercial livestock. Strategies to mitigate clostridial diseases include vaccination to prevent infection or the use of antibiotics after disease is confirmed. Alpha-toxigenic strains of *Clostridium septicum* have been shown to cause spontaneous gas gangrene or atraumatic myonecrosis in humans (Mallozzi et al., 2010), malignant edema in cattle and braxy in sheep (Songer et al., 1996), gangrenous dermatitis in chickens (Li et al., 2010), and clostridial dermatitis in commercial turkeys (Clark et al., 2010). Clostridial dermatitis, also referred to as cellulitis, affects commercial turkey flocks, primarily as they approach market age (Clark et al. 2010). Mortality and plant condemnations result in large economic losses for producers. *C. septicum* is the primary agent associated with dermatitis in commercial turkeys (Tellez et al., 2009). *C. septicum* is a Gram-positive, anaerobic, spore former. The spores are incredibly resilient and long lasting in the environment. When conditions are conducive for growth, the spores will germinate, proliferate, and produce multiple toxins. One in particular, the hemolytic, pore-forming alpha-toxin is the predominant virulence component produced by *C. septicum* (Kennedy et al., 2009). Antibiotics have been therapeutically used after the onset of disease, but with the consumer push for antibiotic-free production, alternatives to antibiotics to control dermatitis in commercial turkey flocks are being investigated. Bacterin-toxoid vaccination programs have been shown to prevent clostridial diseases in other species, including humans (Kotloff et al., 2000). Results from previous field studies indicate that vaccination with an experimental *C. septicum* bacterin-toxoid oil emulsion vaccine reduced dermatitis-associated mortality and antibiotic usage for some commercial turkey flocks, but vaccination was not always efficacious (Thachil et al., 2013; Graham et al., 2019).

The overall purpose of the current project was to optimize CS antigen titer production for an autogenous bacterin-toxoid in a 50L steam-in-place fermentation process and determine the

appropriate vaccine formulation, dosage volume, and route of administration for use in commercial turkey flocks. To improve vaccine efficacy, studies were conducted to optimize the antigenic component of the experimental vaccine and to determine the appropriate antigen to adjuvant ratio, route, and volume for vaccine administration. It was determined that cellular growth phase and hemolytic titer at the time of formalin inactivation played key roles in serum antibody immune response and larger volume vaccine doses produced a higher serum antibody immune response regardless of antigen:adjuvant formulation ratio or route of injection. No significant (P>0.05) differences were found between formulation ratios or between the subcutaneous and tail head injection sites. Additional field studies are underway to assess the protective ability of the optimized bacterin-toxoid antigen at the most efficacious dose volume revealed by the studies reported herein.

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Chapter II. Literature Review

Overview of *Clostridium septicum*-associated dermatitis (cellulitis) and immunogenicity studies in commercial turkeys

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Introduction

Species of the genus *Clostridium* are ubiquitous in soil as well as the normal microflora of humans and animals. Some species of clostridia are pathogenic due to their ability to produce potent exotoxins against which there are no defined host defenses (Wells & Wilkins,1996). In humans and animals, *Clostridium* spp. cause multiple severe diseases requiring therapeutic antibiotics (Singer et al., 2020) or routine vaccinations (Zaragoza et al., 2019). The spore forming ability of clostridia make host-microbe interaction difficult or impossible to control. These spores remain dormant in unfavorable environments for extended periods of time. Upon reintroduction to an environment favorable for growth, such as the nutrient rich tissues of an animal, the bacteria return to a normal growing vegetative state where they quickly replicate and become pathogenic (Clark et al., 2010). Due to the nature of modern turkey production and the practice of housing turkeys on re-used litter in the United States, latent clostridial spores may build up to excessive levels in the housing environment when the litter is of poor quality. This could potentially result in birds coming into contact with pathogenic *C. septicum.* Due to the content of this thesis, *C. septicum* will be the primary focus of this literature review.

Brief overview of clostridial diseases

Diseases caused by pathogenic *Clostridium* spp. have long afflicted both humans and animals. These diseases are caused by the various exotoxins produced inside a host's body, or in incorrectly processed or prepared foods as is the case with *C. botulinum,* with each species producing a unique array of virulence factors (Wells & Wilkins, 1996; Orrell & Melnyk, 2021). *C. botulinum, C. tetani, C. difficile, C. perfringens, C. septicum,* and *C. chauvoei* are a few of the more reported on isolates affecting humans and commercial livestock. *C. tetani and C. difficile* have particular importance for humans due to their increased risk of medical complications and high mortality rate (Enoch et al., 2020). *C. botulinum* is unique among clostridia in that it is capable of producing toxins that accumulate in the environment, such as packaged food and

canned goods, that once ingested are capable of causing disease (Wells & Wilkins, 1996). *C. septicum, C. perfringens, and C. chauvoei* infections in particular account for large economic losses in agriculture. This literature review will focus on *C. septicum* and associated dermatitis in commercial turkeys.

Clostridium septicum

C. septicum is a pathogenic, rod-shaped, motile, anaerobic, spore-forming bacterium capable of causing clostridial myonecrosis in humans and animals (Tran & Myint, 2007; Tellez et al., 2009; Clark et al., 2010). *C. septicum* can be found in soil and litter (Songer et al., 2006), though it is still unknown whether it is a normal member of the microflora of turkeys (Clark et al., 2010). Along with other pathogenic clostridia, *C. perfringens, C. sordelli,* and *C. chauvoei* among others, *C. septicum* has been recovered from emphysematous cellulitis lesions in turkeys (Tellez et al., 2009; Clark et al., 2010). However, *C. septicum* has been identified as the primary etiology of cellulitis in turkeys (Tellez et al., 2009) and has been the focus of disease challenge or immunogenicity studies (Tellez et al., 2009; Thachil et al., 2010; Thachil et al., 2013; Graham et al., 2019). Despite nearly three decades of extensive research, there are still many questions surrounding the portal of entry of *C. septicum* in commercial turkeys and a fully efficacious vaccination strategy has yet to be reported.

C. septicum produces four main exotoxins: the lethal, necrotizing hemolytic alpha-toxin, DNase beta-toxin, hyaluronidase gamma-toxin, and oxygen-labile delta-toxin (Hatheway, 1990). Of these, the hemolytic alpha-toxin has been researched most extensively due to its implication in pathogenesis and ability to cause cellular necrosis (Kennedy et al., 2005; Kennedy et al., 2009a; Kennedy et al., 2009b). The *C. septicum* alpha-toxin is a protoxin similar to the *Aeromonas hydrophila* aerolysin (Ballard et al., 1995) which is released in an inactive state and cleaved by the cell surface receptor furin (Ballard et al., 1992). The alpha-toxin has the ability to bind glycophosphatidylinositol (GPI)-anchored cell surface receptors which enables pore

formation (Melton-Witt et al., 2006). This pore forming ability enables the β-barrel protoxin to lyse host red blood cells (Kennedy et al., 2009a). *In vitro* red blood cell assays confirmed that >95% of observable hemolytic activity is due to the alpha-toxin (Ballard et al., 1992). The pleomorphic nature exhibited by *C. septicum* may play an important role in toxin production and perhaps disease pathogenesis in turkeys. *C. septicum* is hemolytic on agar containing sheep's red blood cells and has the ability to migrate, or "swarm", across agar plates due to a hyperflagellated swarming phase (MacFarlane et al., 2001). Swarming is readily visible on plates containing 1% agar but is inhibited when the agar inclusion reaches 4% (Wilson & Macfarlane, 1996) possibly suggesting that the rigidity of the agar matrix may alter nutrient acquisition by *C. septicum.* This could be of immunological importance due reports that *C. septicum* beta, gamma, and delta toxins are secreted by swarm cells, but alpha toxin is not (Macfarlane et al., 2001). In broth cultures, *C. septicum* alpha-toxin production corresponds to cell phase with peak hemolytic activity achieved as log phase transitions into stationary phase (Ballard et al., 1992; Cortinas et al.,1997). These reports mirror findings from our laboratory as samples from a 50L batch culture of *C. septicum* revealed peak hemolytic activity corresponded with the transition from log phase to stationary phase (unpublished findings). In our experiments, hemolytic activity reduced rapidly as cell concentration (Log_{10} cells/mL) and cell morphology revealed the cell culture moved from logarithmic growth into stationary phase and finally the cell death phase. Furthermore, the swarming phenomenon with low hemolytic titer occurred early in log phase while peak hemolytic toxin was secreted by short rods at late log phase/early stationary phase. These findings are similar to those reported by Ballard et al. (1992) and Cortinas et al. (1997).

In commercial turkeys, vaccination with immunogenic components of the *C. septicum* alpha-toxin have increased serum antibody levels and decreased mortality in laboratory and field studies (Thachil et al., 2013; Graham et al., 2019). The hemolytic ability of *C. septicum* cultures and supernatants is greatest when obtained from cultures containing short rods while

cultures and supernatants from cultures containing swarm cells yielded DNase, hyaluronidase, and neuraminidase toxins (Macfarlane et al., 2001). While the alpha-toxin has been shown to be essential for virulence and is an immunological antigen, non-hemolytic cultures containing the beta, gamma, and delta-toxins are highly toxic to Vero cells (Macfarlane et al., 2001). In addition, recent whole genome sequencing studies have revealed the presence of a potentially novel toxin homolog, *C. septicum* toxin A (CstA), which was conserved across all *C. septicum* genomes evaluated (Thomas et al., 2021). Phylogenetic analysis showed that CstA is closely related to *C. chauveoi* toxin A (CctA), the most efficacious target for vaccines used to prevent blackleg in cattle (Frey et al., 2012; Nicholson et al., 2019). A greater understanding of the relationship between the toxins produced by different *C. septicum* morphologies could provide key insights for producing a more immunogenic and efficacious vaccine.

Other toxigenic clostridial species

C. chauvoei is the primary etiology of blackleg, a disease of cattle and sheep and is closely related to *C. septicum* (Hatheway et al., 1990). *C. chauvoei* is an anaerobic spore-forming, Gram-positive rod that can be found in soil and manure where animals can come into contact with viable spores either through ingestion or through abrasions or cuts in the skin. *C. chauvoei* is classified as a flagellated, motile bacterium. However, a phase variation stage has been reported in which cultures of *C. chauvoei* have produced flagellated motile and non-motile colonies along with aflagellated, non-motile colonies (Tamura et al., 1995). The authors reported that there were no other biochemical or serological differences between the phenotypic variants and the parent strain (*C. chauvoei* strain Okinawa). However, in mouse LD_{50} studies, the aflagellated, non-motile phenotypic variants were less virulent than flagellate motile and flagellate non-motile variants (Tamura et al., 1995). The phase variation stage has been reported in various Gram-negative bacteria but seems to be unique to *C. chauvoei* among Gram-positive bacteria (Tamura et al., 1995). A β-barrel pore forming leucocidin toxin, *C.*

chauvoei toxin A (CctA) has been identified as an important virulence and antigenic factor. An immunization study in guinea pigs utilizing a recombinant CctA and *E. coli* heat labile toxin B (rCctA:LTB) as a peptide adjuvant resulted in increased protection and reduced disease compared to the non-vaccinated control when challenged with spores from a virulent *C. chauvoei* isolate (Frey et al., 2012). Since CctA and CstA, the novel toxin homolog identified for *C. septicum,* appear to be phylogenetically similar (Thomas et al., 2021), immunogenicity studies assessing the protective efficacy of CstA with and without the alpha-toxin against clostridial dermatitis in turkeys would be of interest.

Along with *C. septicum, C. perfringens* has been recovered from clostridial dermatitis afflicted turkeys and chickens (Clark et al., 2010). *C. perfringens* is a non-motile, spore forming, Gram-positive bacillus that is a part of the normal microflora of both agricultural animals and humans. In humans, this bacterium has been associated with disease ranging from mild food poisoning and diarrhea to necrotizing enterocolitis and myonecrosis (Yao & Annamaraju, 2022). Classification and identification of *C. perfringens* is based on the toxins produced by the different subtypes, Type A – Type G (Rood et al., 2018), and these subtypes produce one or more of the following toxins: alpha-toxin (CPA) which is responsible for gas gangrene in humans and animals; beta-toxin (CPB) causes necrotic enteritis in both humans and animals (Uzal et al., 2014); epsilon-toxin (ETX) involved with enterotoxaemia in sheep and goats (Songer et al., 1996); iota-toxin (ITX) associated with enteritis in rabbits (Smedley et al., 2004); enterotoxin (CPE) responsible for human food poisoning and diarrhea (Uzal et al., 2014); and necrotic B-like toxin (NetB) a causative agent of necrotic enteritis in chickens (Keyburn et al., 2010). The NetB toxin was investigated for its role in causing avian necrotic enteritis (NE). A *C. perfringens* NetB mutant, capable of alpha-toxin production and perfringolysin O expression, without NetB production, was constructed. In an NE animal trial, the NetB mutant failed to produce clinical lesions while both the wild type and the mutant containing a $NetB^{+}$ plasmid produced significant levels of disease (Keyburn et al., 2010). Aside from enteric infections, live spore cultures of *C.*

perfringens or *C. septicum* were administered subcutaneously (at 0.5mL, 0.75mL, 1mL, 2mL, or 3mL) to determine if *C. perfringens* was capable of inducing dermatitis (cellulitis) in commercial turkeys (Thachil et al., 2010). Injection with the *C. septicum* spore culture produced mortality and lesions consistent with cellulitis at 24h post-injection with doses of 1mL, 2mL, and 3mL whereas the *C. perfringens* spore culture resulted in mortality with lesions at only the 3mL dose (Thachil et al., 2010). These findings, along with reports of *C. perfringens* recovery from dermatitis lesions, strongly indicate that *C. perfringens* may play a secondary role in the pathogenesis of clostridial dermatitis in turkeys.

C. tetani is a motile, spore forming, anaerobic bacterium that causes the disease tetanus in humans and animals (Cook et al., 2001). Resilient spores of *C. tetani* can be recovered from the manure of animals and are found globally. The disease may afflict persons of any age and results in high mortality rates even when medical intervention is available (van Heyningen, 1968). Toxigenic strains of *C. tetani* may enter the body through deep puncture wounds or the use of non-sterile surgical equipment (WHO, 2017). Once established, the vegetative cells begin to produce tetanolysin, an oxygen-labile hemolysin, that damages localized tissue to create an environment suitable for cellular growth and the neurotoxin tetanospasmin (TeNT) that binds to neural membranes preventing the release of neurotransmitters from the affected neurons (van Heyningen, 1968; Hatheway et al., 1990; Cook et al., 2001). *C. tentani* toxoid vaccines have been utilized for almost 100 years to prevent tetanus in humans and livestock (George et al., 2022). However, *C. tetani* toxoid vaccines do not provide lifelong protection from a single dose. Multiple booster vaccinations are required to maintain protective antibody levels. This suggests that an inactivated vaccine should theoretically be immunoprotective for other clostridial diseases, but the protective threshold provided by a single dose of vaccine may not be sufficient.

C. septicum-associated dermatitis in commercial turkeys

Although the true portal of entry for *C. septicum* in commercial turkeys has not been determined, two theories have been proposed: 1) the inside-out theory and 2) the outside-in theory (Clark et al., 2010). The inside-out theory suggests that *C. septicum* vegetative cells translocate from the gut lumen due to an increase in intestinal permeability and migrate to tissues where the *C. septicum* spores can remain dormant for an extended period. Germination of the *C. septicum* spores occurs due to injury or other event that promotes a hypoxic environment in the subcutis. Alternatively, the outside-in theory suggests that wounds or abrasions become contaminated with *C. septicum* spores that are present in the litter. The anaerobic environment created as a byproduct of tissue damage promotes germination of the *C. septicum* to a rapidly replicating vegetative state. Investigations aimed to define the portal of entry and factors associated with the progression of clostridial dermatitis in turkeys have been briefly described below.

Inside-out theory

The inside-out theory suggests that *C. septicum* within the gut translocates to tissue where the spores remain in a quiescent state until conditions in the tissue support germination and replication. The pathogenesis of *C. chauvoei,* the primary etiology that causes blackleg in cattle, occurs in a similar manner without any indication of injury to the tissue suggesting that the infection is endogenous (Songer et al., 1996). *C. septicum* has been detected in tissue of healthy, asymptomatic turkeys and the spontaneous presentation of clostridial dermatitis in heavy hens and toms may be associated with the rapid growth rates of commercial turkeys as they approach market age (Lighty, 2015). However, attempts to reproduce the inside-out presentation of clostridial dermatitis in turkeys in a research setting have not been successful. For example, intravenous injection with *C. septicum* spore culture has been shown to induce cellulitis in day-of-hatch turkey poults (Tellez et al., 2009). Oral administration of *C. septicum*

spore culture did not lead to disease in healthy 9-week-old turkeys (Thachil et al., 2014). This suggests that damage to the intestinal barrier may be required to permit translocation of *C. septicum*. In another study, dexamethasone, a synthetic corticosteroid used to experimentally immunosuppress and increase intestinal permeability (Vicuna et al., 2015) has been shown to increase dermatitis mortality in commercial turkeys (Huff et al., 2013; Huff et al., 2014) but had no effect in conjunction with oral administration of *C. septicum* spore culture (Thachil et al., 2014).

Commercially reared turkeys are exposed to a plethora of stressors and enteric microbes that impair gut barrier function. It has been theorized that *C. septicum* spores in the lumen of the gut spread hematogenously and can remain dormant in tissue. Damage and bruising of the tissue creates a hypoxic environment that promotes germination of *C. septicum* spores followed by replication and subsequent toxin production. Investigations evaluating the development of cellulitis in healthy turkeys after oral administration of *C. septicum* did not result in disease (Thachil et al., 2014). However, there was no attempt to detect or recover *C. septicum* from the tissue in this particular study. In 2010, the US Department of Agriculture's National Animal Health Monitoring System (NAHMS) evaluated the incidence of intestinal pathology and recovery of *Clostridium* spp. from the gastrointestinal tract of commercial turkeys reared in the United States by clostridial dermatitis outbreak status (USDA, 2012). Results from this study indicated that intestinal pathology was consistent across all flocks evaluated (non-affected; outbreak farm/not currently breaking; outbreak farm/currently breaking) suggesting that increased intestinal permeability is expected in a commercial setting. Furthermore, *C. septicum* recovery from the liver and spleen supports the theory of hematogenous spread. *C. septicum* was also recovered from lesions of clinically affected turkeys or mortalities, but not from the gastrointestinal tract of apparently healthy turkeys. An attempt to detect *C. septicum* in the skeletal muscle of asymptomatic commercial turkeys revealed the presence of *C. septicum* DNA but not *C. septicum* alpha-toxin mRNA (Lighty, 2015). *C. septicum* alpha-toxin has been shown

to cause non-traumatic spontaneous gas gangrene in humans which has be associated with the hematogenous spread of *C. septicum* from the gut (Stevens et al., 1990). Perhaps the presence of toxigenic *C. septicum* in combination with enteric insults and the inherent physical stressors associated with commercial turkey production may promote development of clostridial dermatitis in turkeys. This further highlights the multifactorial nature of *C. septicum*-associated diseases in both humans and livestock.

Outside-in theory

C. septicum spores are extremely resilient to the environmental conditions of the turkey barn and can remain dormant in the environment for extended periods. Although gangrenous dermatitis in broiler chickens may develop because of an endogenous infection, the disease appears to be primarily caused by traumatic injury to the dermis which leads to subcutaneous invasion of microbes (Opengart, 2013). The length of commercial turkey production is much longer than that of broiler chickens. Thus, wound contamination by *C. septicum* during the longer production period of a commercial turkey flock is highly probable (Clark et al. 2010). Following introduction of the pathogen through abraded skin, an injurious event with bruising could create a hypoxic environment in the tissue that promotes *C. septicum* germination and the rapid proliferation of vegetive cells. During replication, the accumulation of *C. septicum* toxins in the tissue causes necrosis, and in severe cases, mortality. Tellez et al. (2009) demonstrated that *C. septicum* is the primary etiology that causes dermatitis in commercial turkeys and this work was further validated by Thachil et al. (2010). To assess the effects of *C. septicum* on morbidity and mortality in neonatal turkey poults, *C. septicum* supernatant, pure culture, or select ratios of *C. septicum* supernatant and convalescent antiserum from *C. septicum* affected turkeys, was subcutaneously administered to turkey poults at day-of-hatch (Tellez et al., 2009). The most notable effects were associated with intravenous injection of live cultures of *C. septicum* (10^8 CFU/bird) which caused lesions consistent with cellulitis and death within 36h. As part of this same study, the subcutaneous injection of formalin inactivated (0.25% formaldehyde

v/v) culture and the cell free supernatant treated with convalescent serum (22°C for 1h) were shown to be incapable of causing mortality, though supernatant without convalescent serum did induce clinical signs of ataxia and incoordination 2h post inoculation (Tellez et al., 2009). From this, the active replication of *C. septicum* and subsequent toxin accumulation could be the cause of high mortality due to overwhelming septicemia. Thachil et al. (2010) evaluated the effects of mortality in 3- and 7-week-old turkeys via subcutaneous challenge with *C. septicum*. Susceptibility appeared to increase with age since there was elevated mortality in 7-week-old turkeys compared to 3-week-old turkeys. The lethality of subcutaneous injections containing live cell culture indicates that a potential portal of entry for *C. septicum* is from the outside-in. More research is needed to determine the primary portal of entry for *C. septicum* associated with clostridial dermatitis outbreaks in commercial turkeys.

Vaccination as a strategy to prevent dermatitis in commercial turkey flocks

Presently, there are no approved vaccines to prevent clostridial dermatitis in commercial turkey flocks. Although there are no commercially available antibiotics specifically approved for treatment of dermatitis in commercial turkeys, antibiotic treatment is commonly required to mitigate an outbreak in a flock (Clark et al., 2010). Penicillin or lincomycin are administered in the drinking water at the onset of disease (Lighty et al., 2016) and continuously provided until there are no additional *C. septicum*-associated mortalities or lesions observed (Clark et al., 2010). However, due to the consumer push for reduced use of human relevant antibiotics in commercially reared poultry, there is a need for efficacious alternatives to antibiotics to control dermatitis in turkeys. Alternatives may include different management practices, vaccination, and/or feed additives that shift the gut microbiota. However, for the purpose of this literature review, experimental vaccines will be the primary focus. Refer to Clark et al. (2010) for a comprehensive review of strategies used to prevent or control dermatitis in commercial turkey flocks in the United States.

Historically, inactivated vaccines have been used to control clostridial diseases in humans and livestock but require multiple doses to establish protective immunity (Zaragoza et al., 2019; Khiav & Zahmatkesh, 2021). To prevent dermatitis in commercial turkeys, subcutaneous vaccination with a *C. septicum* bacterin-toxoid (Tellez et al., 2009; Thachil et al., 2013; Graham et al., 2019) or a bivalent bacterin-toxoid containing *C. septicum* and *C. perfringens* (Thachil et al., 2012) have been evaluated. Since *C. septicum* is the primary etiology for dermatitis in turkeys (Tellez et al., 2009), vaccination with a *C. perfringens* bacterin-toxoid would likely not be effective. Since the *C. septicum* alpha-toxin does not possess the phospholipase C activity of *C. perfringens* alpha-toxin, cross-reactivity between *C. perfringens* and *C. septicum* alpha-toxin is not expected (Ballard et al., 1992). *C. perfringens* may be recovered from dermatitis lesions identified on turkeys, but these are probable secondary contaminants. As a result, inclusion of *C. perfringens* antigenic components would not prevent dermatitis in turkeys. Instead, sufficient concentrations of *C. septicum* antigenic components would be required to produce immunoprotective vaccines.

Subcutaneous administration of an aluminum hydroxide-adjuvated *C. septicum* bacterin-toxoid at hatch increased antibody levels to *C. septicum* in a laboratory trial (Tellez et al., 2009). Oil emulsion-based adjuvants may prolong the immune response due to the depot effect at the site of injection (Aucouturier et al., 2001). Thachil et al. (2013) produced an experimental formalin inactivated water-in-oil bacterin-toxoid vaccine using culture from a single *C. septicum* isolate (UMNCS 106) recovered from clinical breast lesions. The bacterin-toxoid was administered to 6 week-of-age Nicholas White turkeys from a flock with no prior history of dermatitis as a single dose of either 1mL or 2mL. Turkeys were observed for adverse effects from the vaccine for a period of 14 days at which time half of the birds from each group were boosted with an additional 1mL or 2mL dose of the previously described bacterin-toxoid vaccine, respectively. Following an additional 14-day monitoring period, birds from each vaccinated group, and immunologically naive birds, were subcutaneously challenged with a *C.*

septicum culture. Vaccination with the *C. septicum* bacterin-toxoid prevented mortality 24h post-challenge compared to non-vaccinated counterparts. However, there were no statistical differences in serum antibody levels between the 1mL or 2mL dose. In a field study, a 1mL dose of the vaccine lowered *C. septicum*-associated mortality and reduced penicillin pack usage compared to the non-vaccinated group. In another study, subcutaneous vaccination with a *C. septicum* bacterin-toxoid water-in-oil vaccine at hatch induced markedly higher antibody levels at 5 weeks post-vaccination compared to aluminum hydroxide or mannosylated chitosan adjuvant (Graham et al., 2019). Vaccination with this *C. septicum* bacterin-toxoid water-in-oil vaccine in 8-week-old commercial turkeys elevated *C. septicum* antibody levels at 12 and 16-20 weeks-of-age and reduced *C. septicum*-associated mortality and penicillin usage on some of the farms evaluated. Taken together, vaccination with a *C. septicum* bacterin-toxoid oil emulsion vaccine may prevent *C. septicum*-associated dermatitis in commercial turkey flocks but may require the intermittent use of penicillin to control the disease. Thus, the use of the vaccine should be considered on a farm-to-farm basis due to the incomplete protection observed on select farms.

There has been an attempt to generate a recombinant, noncytolytic *C. septicum* alpha-toxin to prevent *C. septicum*-associated dermatitis in turkeys (Lancto et al., 2014). Although the recombinant vaccine proved to be efficacious in a challenge study, to our knowledge, no further attempts to evaluate this vaccine in a commercial setting have been conducted. The research presented here indicates that the current single administration of a *C. septicum* bacterin-toxoid commonly employed by the industry does not provide fully protective immune response in commercial turkeys. A recombinant vaccine that could be administered in the drinking water at multiple time points could provide a cost-effective strategy to maintain elevated serum antibody levels to the *C. septicum* alpha-toxin in commercial turkey flocks.

Conclusion

Clostridium septicum is ubiquitous in the environment of commercial turkey facilities. The presence of *C. septicum* in the gastrointestinal tract, tissues, and organs of healthy, asymptomatic turkeys indicates frequent exposure to *C. septicum* during commercial production. With antibiotic treatment as the single strategy to treat affected flocks, there is a need for research to identify sustainable and effective prophylaxis for clostridial dermatitis in turkeys. Immunization with inactivated vaccine requires multiple doses and is not economically feasible for commercial turkey producers. Our laboratory determined that the hemolytic activity of *C. septicum* cultures at the time of formalin inactivation alters the humoral immune response of *C. septicum* bacterin-toxoid in turkeys. Moreover, vaccination with a live-attenuated *C. septicum* strain followed by administration of a *C. septicum* bacterin toxoid oil emulsion vaccine could be evaluated as a method to mitigate dermatitis on farms that have reoccurring outbreaks or in antibiotic free production systems. Further research is needed to define the true portal of entry and pathogenesis of *C. septicum* and to identify effective antibiotic alternatives to control dermatitis in commercial turkeys.

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Chapter III. Evaluation of *Clostridium septicum* hemolytic activity, administration route, and dosage volume of a clostridial dermatitis (cellulitis) bacterin-toxoid on humoral immune response in commercial turkeys

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Abstract

Clostridial cellulitis or dermatitis affects commercial turkey flocks, primarily as they approach market age. In the field, this disease has been effectively controlled with antibiotics, but alternatives to antibiotics are needed. Bacterin-toxoid vaccination programs have been shown to prevent clostridial diseases in other species, including humans. Results from previous field studies indicate that vaccination with an experimental whole-cell *Clostridium septicum* (CS) bacterin-toxoid oil emulsion vaccine reduced clostridial dermatitis-associated mortality and antibiotic usage for some commercial turkey flocks, but vaccination was not always efficacious. To improve vaccine efficacy, studies were conducted to optimize the antigenic component of the experimental vaccine and to determine the appropriate antigen to adjuvant ratio, route, and volume for vaccine administration. It was determined that the phase of a cell culture at time of formalin inactivation played a key role in serum antibody titer and larger volume vaccine doses produced higher serum antibody immune response regardless of antigen:adjuvant formulation ratio or route of injection. No significant differences ($P>0.05$) were found between formulation ratios or between the subcutaneous and tail head injection sites. Based on these results, we propose to look further into the relationship between cell culture phase and antigenic components produced by CS under different culture conditions.

Introduction

CS is the primary causative agent associated with clostridial dermatitis in commercial turkeys (Tellez et al., 2009). Mortality and plant condemnations result in large economic loss for producers (Clark et al., 2010). CS is a Gram-positive, anaerobic, spore-former capable of remaining dormant in the environment for prolonged periods of time. Cleaning and disinfection have proven insufficient as a strategy for eliminating clostridial spores from the farm environment. When conditions are conducive for growth, the spores will germinate, proliferate and produce multiple toxins. One in particular, the hemolytic, pore-forming alpha-toxin is the predominant virulent component of CS (Kennedy et al., 2005). Antibiotics have been

therapeutically used after onset of disease, but with the consumer push for antibiotic-free production, the turkey industry has no commercially available option to prevent clostridial dermatitis-related mortality in commercial turkeys. Although penicillin is used to reduce CS-associated mortality under field conditions, vaccination with a whole-cell CS bacterin-toxoid has been shown to be immunologically efficacious and reduce clostridial dermatitis-related mortality in commercial turkeys (Thachil et al., 2010; Graham et al., 2019).

Previously, we demonstrated that immunization with an oil-adjuvated bacterin-toxoid increases serum antibody response to CS alpha-toxin (detected by ELISA) under laboratory and commercial conditions (Graham et al., 2019). However, vaccination was not always efficacious. This could be due to a multitude of factors. Previous research has shown that the lethal, necrotizing, hemolytic alpha-toxin is essential for virulence (Kennedy et al., 2009) and pathogenicity and is produced by short rods (Macfarlane et al., 2001). However, samples obtained from cultures containing predominantly swarming cell morphology were non-hemolytic to red blood cells but were toxic to Vero cells due to excretion of DNase, hyaluronidase, and neuraminidase (Macfarlane et al., 2001). Additionally, the volume of the vaccine injection has been shown to affect efficacy of an experimental CS bacterin-toxoid vaccine with higher doses (Thachil et al., 2013). Here an attempt was made to optimize the hemolytic ability of the two CS isolates used in our experimental bacterin-toxoid vaccine and to measure the effects, if any, on serum antibody titer when immunized with experimental vaccine containing high or low hemolytic tittered antigen in a high or low dose. Thus, we hypothesized that 1) vaccination with high hemolytic titer antigen (CS bacterin-toxoid) will increase antibody response to the CS alpha-toxin compared to low hemolytic titer antigen and 2) route of administration, subcutaneous vs. tail head, and volume administered would improve immune response. The purpose of the present study was to optimize CS antigen titer and determine an appropriate vaccine formulation, dosage volume, and route of administration.

Materials and methods

Bacterial isolates

The two CS isolates (CS2, CS3) used in these experiments were obtained from fluid emphysematous lesions from clostridial dermatitis-affected turkey carcasses and confirmed as CS using commercial anaerobic isolation panels (Tellez et al., 2009).

In vitro Optimization (Exp 1)

CS Morphology and Hemolytic Activity

In Exp 1, an attempt was made to optimize CS alpha-toxin production under large scale fermentation conditions. A 50L fermenter was utilized to determine time and temperatures effects on the hemolytic toxin titer of a co-culture of isolates CS2 and CS3. To obtain the initial inoculum for 50L batch fermentation, CS2 and CS3 were grown separately overnight in 2L anaerobic cultures of tryptic soy broth (TSB) with sodium thioglycolate at 37°C. Hemocytometer counts of 24h turbid cell cultures show 2L seeder stock reaches ~3-5x10^8 cells/mL. This 4L of turbid culture was used to seed 46L of TSB/thio media in a steam-in-place fermenter bringing the final volume to 50L. Final temperature, pH, duration of fermentation, and hemolytic units (HU) were recorded for nine separate 50L fermentations. The pH of was monitored continuously in real time using an in-line multi-parameter transmitter (M300 Transmitter; Ohaus, Mettler-Toledo Ind, Columbus, OH). A tenth 50L co-culture of CS2 and CS3 was monitored periodically for pH, cell morphology, growth phase, and hemolytic activity. Collected samples were subjected to microscopy for cell counts, gross morphological changes, and hemolytic red blood cell (RBC) assays (Graham et al., 2019) to identify connections between cellular phase and hemolytic ability.

In vivo (Exp 2 and Exp 3):

General antigen production and vaccine preparation

To produce the bacterin-toxoid antigen, CS isolates were grown overnight in a steam-in-place fermenter in TSB with sodium thioglycolate (0.5%), media pH adjusted to 8.0 prior to steam cycle, and inactivated at select times with formalin to achieve a final concentration of 0.25%. Cell concentration was confirmed microscopically, and toxin production was confirmed via RBC assay to determine antigen titer as described above. CS bacterin-toxoid was combined with a water-in-oil adjuvant, Seppic Montanide 71 R VG, at a ratio of 30:70 antigen/adjuvant unless stated otherwise. The homogenization technique utilized in this experimental vaccine was performed as previously reported (Graham et al., 2019).

Antigen comparison *(Exp 2)*

To compare serum antibody response to high or low CS hemolytic-titer antigen, separate 50L batches of CS were grown to a hemolytic titer ranging from 64 HU to 1024 HU. The antigen was combined with Seppic Montanide 71 R VG adjuvant, as previously described. Cultures were mixed during incubation, excluding group 5 antigen (128 HU Static), which was incubated statically. At 6 weeks-of-age, female Nicholas poults (n=140) were transferred to the University of Arkansas JKS Poultry Health Laboratory. The experimental vaccine was administered at 7 weeks-of-age at a 0.5mL dose, subcutaneously in the nape of the neck. Treatment groups (n=20/group) were as follows: 1) non-immunized control, 2) 128 HU, 3) 512 HU, 4) 1024 HU, 5) 128 HU Static, 6) Combined 1024 HU/64 HU antigens at 1:1 ratio, 7) Combined 64 HU/512 HU antigens at 5:1 ratio. Blood was collected from the brachial vein and serum was used to determine pre-immunization serum antibody levels to CS alpha-toxin at 7 weeks-of-age, as measured by ELISA, and evaluated weekly up to 12 weeks-of-age (5 weeks post-vaccination).

Vaccine formulation, volume, and route of administration (Exp 3)

The antigen from Exp 2, Treatment Group 3 (512 HU), was used for this experiment based on improved humoral immune response to CS alpha-toxin. The vaccine was produced at two formulation ratios: 30% antigen to 70% adjuvant or 50% antigen to 50% adjuvant. These two formulations were administered in a low (0.5mL) or high (1mL) dose and via two injection routes: subcutaneously in the nape of the neck (SQ) or intramuscularly in the tail head. At 5 weeks-of-age, female Nicholas poults (n=~189) were transferred to the University of Arkansas JKS Poultry Health Laboratory. Treatment groups included: 1) non-vaccinated control; 2) SQ, 0.5mL dose, 30:70 ratio; 3) SQ, 1mL, 30:70; 4) SQ, 0.5mL, 50:50; 5) SQ, 1mL, 50:50; 6) tail head, 0.5mL, 30:70; 7) tail head, 1mL, 30:70; 8) tail head, 0.5mL, 50:50; and 9) tail head, 1mL, 50:50. The vaccine was administered at 6 weeks-of-age. Blood was collected from the brachial vein (n=20-21/group) and serum was used to determine pre-immunization serum antibody levels to CS alpha-toxin, as measured by ELISA, and evaluated weekly up to 12 weeks-of-age (6 weeks post-vaccination).

Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

The indirect enzyme-linked immunosorbent assay (ELISA) used to measure turkey humoral antibody levels against the CS alpha-toxin has been previously described (Tellez et al., 2009). Briefly, serum was collected prior to inoculation with experimental bacterin-toxoid vaccine and weekly thereafter to measure any change in serum antibody levels. The assay was performed in a manner consistent with methods previously described (Roberts et al., 1987: Ameiss et al., 2004: Hang'ombe et al., 2005). Samples were read on a commercial microplate reader (BioTek MQX200, BioTek Instruments Inc., Winooski, VT.) at an absorbance of 450 nm. Absorbance values for the positive control, negative control, and experimental samples were used to calculate the sample to positive control ratios (S/P ratios) (Brown et al., 1991; Davies et al., 2003).

Institutional Animal Care and Use

This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville, under protocol #21096.

Statistical Analysis

S/P antibody titers are reported as the mean ± standard error. Antibody titer data were subjected to analysis of variance as a completely randomized design using the JMP Pro 15 to determine significant (P<0.05) differences. Means were further separated using Tukey HSD when appropriate. HU reported as an average of 4 replicates.

Results

Exp 1 Results

Exp 1 compared the hemolytic activity of ten separate 50L fermentations at temperatures ranging from 37.2°C to 43.3°C and incubation durations from 7h to 44h (Table 1). Batches that were stirred during incubation and incubated at temperatures ranging from 38.1°C to 41.1°C resulted in fermentate with relatively high hemolytic titer (64-128 HU). However, an incubation temperature of 43.3C negatively impacted HU (16 HU). With this particular vessel, 37.2°C produced fermentate with the highest hemolytic titer of 512 HU. Incubation duration appeared to have less of an effect than temperature as both 15h and 44h incubations produced relatively high hemolytic units (128 HU and 64 HU, respectively) and no relationship was observed from these experiments between incubation length and hemolytic activity. The tenth fermentation was sampled periodically from time of inoculation to time of formalin inactivation and these sample analyses revealed that hemolytic activity was tied to cell phase (Figure 1). It was determined that toxin production *in vitro* correlated with log phase cell growth and the peak hemolytic titer of 1024 HU coincided with the stationary phase, 6.25h post-inoculation. Cell counts revealed that a drop in pH occurred during log phase cell growth. Microscopy showed the presence of swarm

cells similar to those previously reported by Macfarlane et al. (2001) approximately 2h prior to peak toxin production or ~5.5h post-inoculation (data not shown). Swarm cells were not present during peak toxin production, 6.25-6.5h post-inoculation, instead short motile rods, either singly or in short, multisegmented chains, were the prevalent cell morphology observed in conjunction with peak hemolytic activity. Hemolytic titer decreased rapidly during cell death, falling to 512 HU at 7h post-inoculation (Figure 1). A 500mL sample was removed from the 50L fermenter prior to formalin inactivation at 7h and kept at 37°C for further analysis. Hemolytic results from this sample showed a continual decrease of hemolytic titer, 128 HU at 24h and 64 HU at 48h of incubation (data not shown).

Exp 2 Results

In Exp 2, ELISA results from the pre-vaccination and 1-week post-vaccination serum analyses revealed no significant differences in humoral immune response to CS alpha-toxin across all groups (Table 2). Beginning at 2-weeks post-vaccination, Group 3 (512 HU) showed a significant ($P<0.05$) increase in immune response compared to the non-vaccinated control and the group vaccinated with the higher hemolytic titer 1024 HU antigen. This trend continued throughout the study, and at 6-weeks post-vaccination, the 512 HU and combined 5:1 (64 HU:512 HU) antigen elicited significantly *(*$P<0.05$) higher serum antibody levels against the CS alpha-toxin than the higher titer 1024 HU antigen and the Combined 1:1 antigen. The 1024 HU and Combined 1:1 antigen did not produce significantly higher serum antibody titers than the non-vaccinated control. The cumulative immune response for each treatment group is presented in Figure 2. The 512 HU and static 128 HU produced the highest cumulative immune response compared to all other treatment groups.

Exp 3 Results

After comparing administration route, dose volume, and vaccine formulation in Exp 3, it was determined that the 1mL dose elicited a numerically higher, but not significant, serum antibody

titer to CS alpha-toxin than the 0.5mL dose at 6-weeks post-vaccination (Table 3). Additionally, from 3-weeks post-vaccination to study termination at 6-weeks post-vaccination, the SQ 50:50 group that received the 1mL dose elicited a significantly (P<0.05) higher immune response than the 0.5mL counterpart, as measured by ELISA. There were no significant differences between antigen/adjuvant ratio or route of inoculation. The cumulative immune response for each treatment group is presented in Figure 3. The SQ 30:70 1mL and SQ 50:50 1mL produced the highest cumulative immune response compared to all other treatment groups.

Discussion

Although virulence of CS in turkeys has been attributed to the CS alpha-toxin (Kennedy et al., 2009; Tellez et al., 2009), hemolytic ability is not the only metric to consider when investigating antigens for potential vaccine use, possibly due to the presence of the hemolytic delta-toxin. This is consistent with previous reports stating that CS produces two hemolysins; an oxygen-labile delta-toxin and an oxygen-stabile alpha-toxin (Howard Moussa, 1958; Ballard et al., 1992) Delays between obtaining samples and performing hemolytic assays have been shown to decrease the titer of the delta-toxin. However, keeping fermentate at a low, unadjusted pH seems to mitigate this degradation, and the addition of sodium thioglycolate is capable of reactivating the previously oxidation inactivated CS delta-toxin (Howard,1953; Moussa, 1958). The use of sodium thioglycolate as a reducing agent in our culture media may be increasing the hemolytic titer of the delta-toxin in our assays. Our findings show when producing a CS bacterin-toxoid vaccine, cellular growth phase at the time of formalin inactivation can have a significant impact on humoral immune response which could be due to various toxins produced at different stages of cellular development. Recently, a putative cytolysin gene termed *Clostridium septicum* toxin A (CstA) was identified through genome mapping of five CS isolates (Thomas et al., 2021). A similar gene from *Clostridium chauvoei* (CctA) has been shown to be a protective antigen against blackleg (Nicholson et al., 2019) Interestingly, the highest HU titer CS antigen did not induce an immune response to CS alpha toxin significantly higher than the non-

vaccinated control. Thus, this rejects our null hypothesis that the highest hemolytic titer antigen would result in the highest serum antibody titer in response to the hemolytic toxin.

These results, in addition to previous studies (Thachil et al., 2013), revealed that larger dose volumes of a CS bacterin-toxoid oil emulsion vaccine may increase serum antibody titers to the CS alpha-toxin. However, our investigation showed no significant differences between the subcutaneous and tail head vaccine administration route at either the 30:70 or 50:50 antigen/adjuvant ratios. This indicates that either vaccine administration route could be considered when implementing a vaccination program.

Experiments were conducted to determine optimal hemolytic toxin producing growth conditions of CS. Bacterial cultures were sampled at different timepoints and hemolytic titer and cell counts were assessed. It was found that toxin production corresponded with increasing cell/mL numbers, peaked at stationary phase, and decreased as cultures entered cell death phase. Antigen was produced from log, stationary, and cell death phase cell cultures to evaluate the effect of the following parameters on immune response to CS alpha-toxin in commercial turkeys: 1) antigen growth phase and hemolytic activity at the time of formalin inactivation, 2) antigen to adjuvant ratio, 3) vaccination route (subcutaneous/nape of neck, intramuscular/tail head), and 4) administration volume. The growth phase of the bacterial culture and pH of the culture media at time of formalin inactivation were shown to have significant influence on hemolytic ability. Immunogenicity was markedly impacted by all parameters evaluated under laboratory conditions, with larger volumes and subcutaneous administration proving most efficacious.

Conclusion

In conclusion, our results from 50L batch cultures have shown hemolytic ability is tied to the growth phase and morphology. Previous reports showed that hemolytic titer of a CS bacterin-toxoid vaccine is an important component of the humoral immune response (Kennedy et al., 2009; Thachil et al., 2013) which is similar to the results in the present study. However,

hemolytic titer is not the only metric to consider when producing a CS bacterin-toxoid vaccine. Currently, our laboratory is evaluating production of a temperature adapted strain of CS that could be given as a live prime immunization.

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Tables and Figures

Table 1. Effect of fermentation conditions on hemolytic CS toxin production (Exp 1)

Lot	Temperature (°C)	pH	Hours post-inoculation	HU[1]
1	43.3	6.02	24	16
2	43.3	6.00	29	16
3	38.6	6.91	44	64
4	39.4	6.26	20	64
5	41.1	6.26	24	64
6	38.1	5.99	24	128
7	38.1	5.80	24	128
[2]8	38.9	6.03	25	32
9	37.8	5.99	15	128
[3]10	37.2	6.28	7	512

[1]Hemolytic units (HU); n=4 replicates for *in vitro* hemolytic assay for each lot
[2]Only fermentation in which culture was statically incubated. All other cultures stirred at 30hz during incubation
[3]Sampled periodically from initial seeding of 50L fermenter until time of formalin inactivation 7h post-inoculation. See Figure 1 for batch 10 growth curve

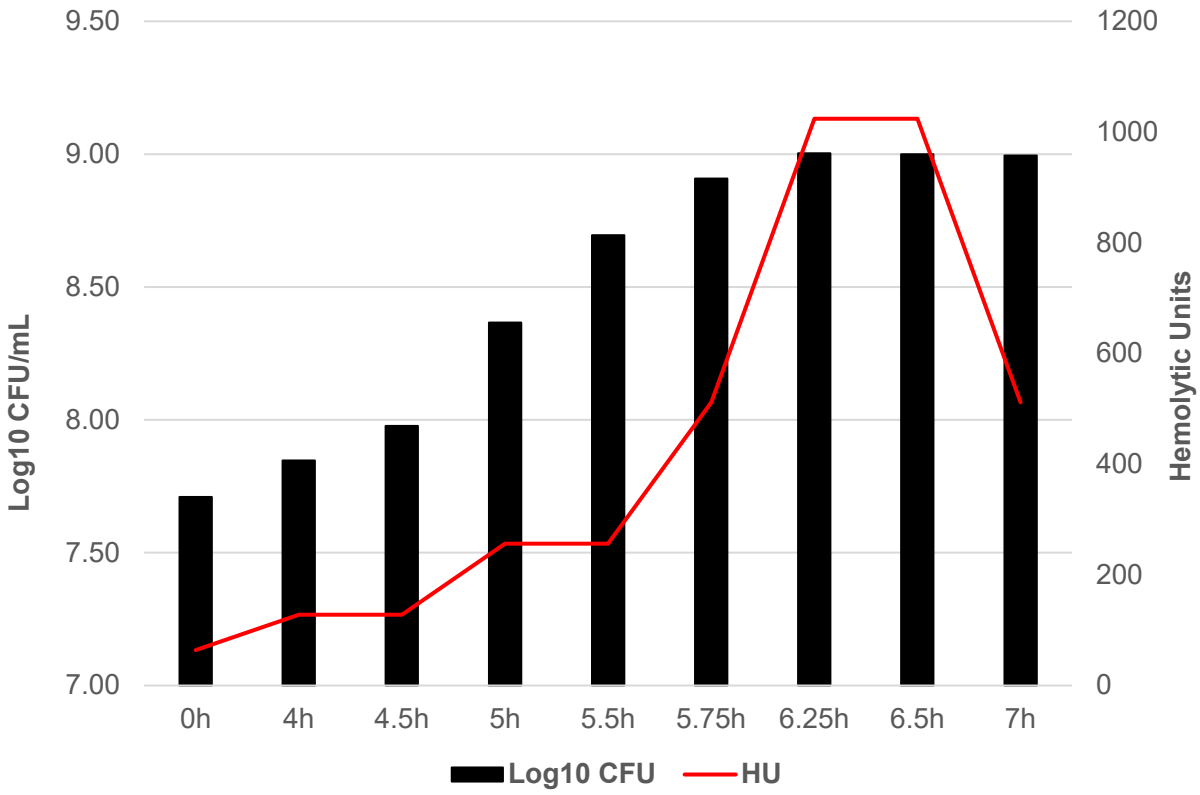

Figure 1. 50L fermentation showing Log_{10} CFU (black bars) and hemolytic titer (red line) of Lot 10 from 0-7h post-inoculation (Exp 1). 4L turbid culture containing ~$3x10^8$ cells/mL was used to inoculate the 46L of TSB/thio. A subsample was collected from the sampling port at the time of inoculation (0h) and at 4h, 4.5h, 5h, 5.5h, 5.75h, 6.25h, 6.5h, and 7h post-inoculation.

Table 2. Comparison of hemolytic titer antigen on humoral immune response at 0-5 weeks post-vaccination (Exp 2)

		Post-vaccination				
Treatment	**Pre-Immunization**	**1-week**	**2-weeks**	**3-weeks**	**4-weeks**	**5-weeks**
Non-vaccinated Control	-0.063 ± 0.003	0.003 ± 0.010	-0.021 ± 0.014^{c}	-0.065 ± 0.009^{d}	-0.031 ± 0.004^{d}	-0.045 ± 0.005^{e}
128 HU	-0.068 ± 0.004	0.002 ± 0.009	0.097 ± 0.034^{abc}	0.182 ± 0.050^{bc}	0.196 ± 0.048^{bcd}	0.358 ± 0.085^{abcd}
512 HU	-0.068 ± 0.004	-0.004 ± 0.010	0.233 ± 0.056^{a}	0.481 ± 0.097^{a}	0.435 ± 0.062^{ab}	0.669 ± 0.086^{a}
1024 HU	-0.063 ± 0.007	-0.009 ± 0.007	0.034 ± 0.019^{bc}	0.145 ± 0.052^{bcd}	0.087 ± 0.019^{cd}	0.118 ± 0.031^{de}
128 HU Static	-0.095 ± 0.003	-0.039 ± 0.006	0.133 ± 0.059^{abc}	0.374 ± 0.085^{ab}	0.546 ± 0.132^{a}	0.485 ± 0.122^{abc}
Combined 1:1	-0.093 ± 0.003	-0.024 ± 0.011	0.100 ± 0.034^{abc}	0.120 ± 0.041^{cd}	0.230 ± 0.061^{bcd}	0.233 ± 0.044^{cde}
Combined 5:1	-0.048 ± 0.001	-0.048 ± 0.005	0.045 ± 0.016^{bc}	0.170 ± 0.041^{bcd}	0.317 ± 0.058^{abc}	0.566 ± 0.094^{ab}

Data expressed as mean ± standard error
ANOVA was used to determine significant (P<0.05) differences and further separated using Tukey HSD
$^{a-e}$ Differing letters within columns show significant (P<0.05) differences

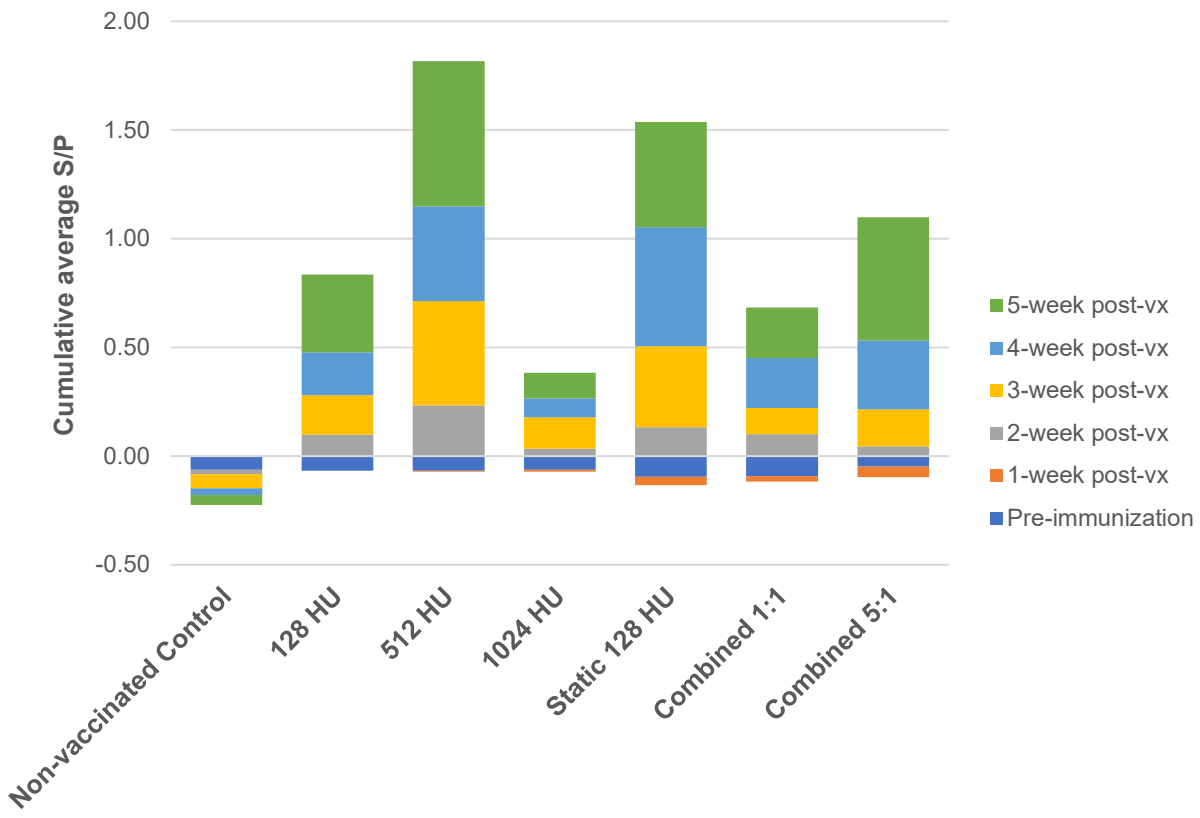

Figure 2. Cumulative average serum antibody titer at 0-5 weeks post-vaccination against *Clostridium septicum* (CS) alpha-toxin with variations of an experimental CS bacterin-toxoid vaccine (Exp 2). Antibody levels as measured by ELISA (S/P) are plotted as a cumulative average. The ELISA absorbance value obtained for the positive control, negative control, and experimental samples were used to calculate the sample to positive control ratio for each week evaluated. Abbreviations are as follows: HU = hemolytic unit titer of CS culture at time of formalin inactivation, Combined 1:1 = 1024 HU/64 HU antigen at 1:1 ratio, and Combined 5:1 = 64 HU/512 HU antigen at 5:1 ratio.

Table 3. Effect of vaccine formulation, dosage volume, and administration route on humoral immune response at 3-6 weeks post-vaccination (Exp 3)

	Post-vaccination			
Treatment	**3-weeks**	**4-weeks**	**5-weeks**	**6-weeks**
Non-vaccinated Control	-0.01 ± 0.00^{c}	-0.03 ± 0.01^{c}	-0.24 ± 0.03^{c}	-0.03 ± 0.02^{c}
SQ 30:70 0.5mL	0.15 ± 0.04^{abc}	0.38 ± 0.08^{abc}	0.37 ± 0.11^{ab}	0.94 ± 0.15^{ab}
SQ 30:70 1mL	0.25 ± 0.05^{a}	0.70 ± 0.12^{a}	0.71 ± 0.11^{a}	1.23 ± 0.15^{a}
SQ 50:50 0.5mL	0.08 ± 0.04^{bc}	0.26 ± 0.12^{bc}	0.13 ± 0.10^{bc}	0.36 ± 0.17^{bc}
SQ 50:50 1mL	0.27 ± 0.07^{a}	0.71 ± 0.14^{a}	0.82 ± 0.17^{a}	1.16 ± 0.18^{a}
Tail Head 30:70 0.5mL	0.07 ± 0.02^{bc}	0.33 ± 0.08^{abc}	0.15 ± 0.06^{bc}	0.50 ± 0.08^{bc}
Tail Head 30:70 1mL	0.17 ± 0.04^{ab}	0.51 ± 0.12^{a}	0.48 ± 0.13^{ab}	0.90 ± 0.17^{a}
Tail Head 50:50 0.5mL	0.17 ± 0.04^{ab}	0.33 ± 0.04^{abc}	0.56 ± 0.11^{ab}	0.89 ± 0.11^{ab}
Tail Head 50:50 1mL	0.20 ± 0.04^{ab}	0.61 ± 0.11^{ab}	0.52 ± 0.08^{ab}	1.18 ± 0.15^{a}

Data expressed as mean ± standard error
ANOVA was used to determine significant (P<0.05) differences and further separated using Tukey HSD
^{a,b,c} Differing letters within columns show significant (P<0.05) differences

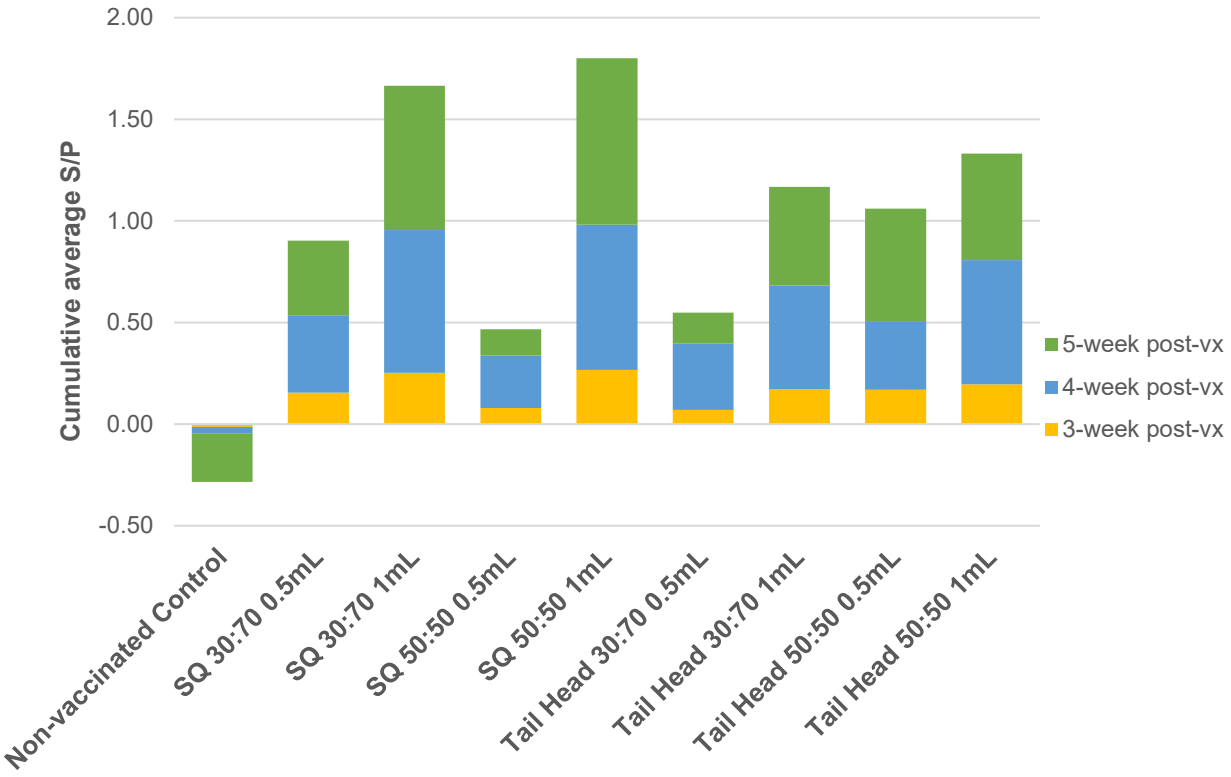

Figure 3. Cumulative average serum antibody titer at 3-5 weeks post-vaccination against *Clostridium septicum* (CS) alpha-toxin with variations of an experimental CS bacterin-toxoid vaccine (Exp 3). Administration of vaccine was either subcutaneously (SQ) in the nape of the neck or intramuscularly in the tail head with 0.5mL or 1mL of experimental CS bacterin-toxoid vaccine formulated at 30:70 or 50:50 antigen/adjuvant ratio at 6-weeks-of-age. Antibody levels as measured by ELISA (S/P) are plotted as a cumulative average. The ELISA absorbance value obtained for the positive control, negative control, and experimental samples were used to calculate the sample to positive control ratio for each week evaluated.

Chapter IV. Thesis Conclusion

Clostridial dermatitis in turkeys is a multifaceted problem. *Clostridium septicum* produces exotoxins that perform roles that are yet to be completely understood in the pathogenesis of clostridial dermatitis. Following the removal of antibiotic therapies, vaccination will be the only strategy to mitigate this disease in commercial turkey flocks. It is essential to identify efficacious alternatives to antibiotics. Our laboratory has investigated the efficacy of hemolytic toxins as antigenic components in an experimental *C. septicum* bacterin-toxoid vaccine. It was found that cellular morphology and the hemolytic toxin titer at time of formalin inactivation affected immunogenicity. The highest hemolytic toxin titer antigen (1024 HU) resulted in the lowest antibody titer levels as measured by ELISA. Toxin production at this level only lasted a brief time, 20 minutes, before halving to 512 HU. Bacterin-toxoid produced from this low titer antigen resulted in a higher humoral antibody response. Vaccine formulation (antigen:adjuvant ratio), administration route, and dosage volume were also evaluated. No significant differences were observed between a subcutaneous or an intramuscular inoculation route or between vaccine formulations when administered at the same dosage volume. However, when comparing similar formulation and route of administration, larger doses elicited greater humoral antibody responses to *C. septicum* alpha-toxin in commercial turkeys. Potential strategies to improve immunoprotection may include vaccination with a live attenuated strain of *C. septicum* prior to immunization with an inactivated bacterin-toxoid. Additionally, recent findings revealed the presence of a potentially novel toxin homolog, one that may be a significant antigenic target, but further research is required.

Appendix

DIVISION OF AGRICULTURE
RESEARCH & EXTENSION
University of Arkansas System

To: Billy Hargis
Fr: Beth Kegley - Ag-IACUC, co- Chair
Date: March 22nd, 2021
Subject: IACUC Approval
Expiration Date: March 18th, 2024

The Division of Agriculture Institutional Animal Care and Use Committee (Ag-IACUC) has APPROVED your protocol # 21096 *Evaluation of the effect of an autogenous Clostridium septicum bacterin-toxoid vaccine under laboratory conditions - II.*

In granting its approval, the Ag-IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the Ag-IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond March 18th, 2024 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy, the Ag-IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez-Isaias, Christine Vuong, Danielle Graham, Callie McCreery Selby, Makenly Coles, Cheryl Lester, Roberto Senas Cuesta, Aaron Forga, Lesleigh Beer, Thaina Barros, and Lucas Graham. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The Ag-IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

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