Characterization of the anti-Clostridial effects of a novel probiotic and its effectiveness in the control and prevention of necrotic enteritis

Thesis

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Abstract

Necrotic enteritis is an enteric disease primarily caused by overgrowth of *C. perfringens* (CP) in the small intestine following a variety of predisposing factors. The objective of this study was to determine if a novel probiotic showed anti-clostridial effects, survived pelleting temperatures and the varied environment of the gastrointestinal tract (GIT), and if anti-clostridial effects were retained through the GIT. The probiotic was tested against 9 strains of CP to determine overarching anti-clostridial effect. The probiotic suppressed all 9 strains of CP significantly (p-value<0.05) when CP inoculated media was overlayed onto a pre-grown colony of probiotic and zones of inhibition measured. Next, probiotic efficacy was compared against common antibiotics and other bacterial isolates. Two of the four antibiotic treatments, penicillin (0.0625mg/mL) and metronidazole (0.05mg/mL), two of the bacterial isolates, and the candidate probiotic were all able to reduce CP growth, with the candidate probiotic outperforming the bacterial isolates (p-value<0.001) and metronidazole (p-value$=0.007$). The CP strain showed resistance to the other two antibiotic treatments, BMD (0.022mg/mL) and avilamycin (0.05mg/mL).

A germination and sporulation assay was completed to ensure spores could germinate at the internal body temperature of chickens and sporulate to survive pelleting. A lack of significant change (p-value$=0.096$) in cell recovery was indicative of the probiotic's ability to germinate to colonize the gut and sporulate to endure pelleting. Interaction of the probiotic candidate with 18 other common enteric residents of the microbiome in poultry were observed in a streak plate assay, in search of potential inhibition. Of the 18 additional strains tested against the probiotic, three strains of *E. cecorum* were significantly inhibited by the probiotic based on Chi^2 analysis. A gastrointestinal survival assay was performed mimicking the crop, proventriculus, and

intestines to ensure the probiotic could survive the gastrointestinal tract. When spores recovered from each section of the GIT were compared, the final concentration was statistically lower than the initial (p-value=0.018) with a 0.35-Log_{10}-fold reduction in the recovered CFU reaching the small intestine. The survival assay was repeated with the addition of 10^8 CFU of CP strain TXAM 020410 in the small intestines portion of the assay to determine if the efficacy of the anti-clostridial properties was maintained. Using a 10^6 dose of spores, the reduction in CP was approaching significance at p-value=0.112 but saw a reduction of nearly 1-Log_{10} CFU of recovered CP. The results of these experiments indicate the probiotic is a candidate for treatment and control of necrotic enteritis due to its broad anti-clostridial properties and resilience in harsh environments.

One of the primary factors that goes into determining if a bacteria would function well as a probiotic, is viability. To ensure the resilient nature displayed *in vitro* was maintained in live birds, a study was developed to ensure the persistence of the spores while in the GIT of a chicken. Two treatments were established, the gavage treatment received a single dose of 10^6 spores on day of hatch and the feed treatment received a consistent supply 10^6 spores per gram of feed. Beginning 24 hours after the initial dose, and every subsequent 24 hours for 5 days, 10 birds per treatment were euthanized and had their crop, ileum, and ceca aseptically collected. 12 pooled fecal samples per treatment per day were also collected to observe changes in spore passage. At all sampled time points (24, 48, 72, 96, and 120 hours after initial dosing) and across all sampled locations, the group consistently fed the spores had a significantly higher (p-value<0.01) spore recovery than the gavaged group and more consistent recovery across all locations in the gut. This suggested the probiotic's ability to complete the life cycle of

germination and sporulation, showing they are active in the GIT of broilers from day of hatch and must be fed on a consistent basis to maintain their presence.

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Chapter 1.

Introduction

Necrotic enteritis (NE) is an enteric disease that is having an increasing economic impact on the poultry industry. While, historically, it was well controlled by antibiotics provided at sub-therapeutic levels in feed, due to both federal and public pressures, the use of antibiotics in the feed has been stopped by many poultry producers. This has resulted in increasing pressure to control the disease with non-antibiotic methods. Necrotic enteritis is a short-term disease that impacts individual birds for 48-72 hours but may roll through a flock, barn, or facility more chronically. The causative agent for NE infection is *Clostridium perfringens*, an opportunistic, anaerobic, spore-forming bacteria that normally resides in the gastrointestinal tract (GIT) of poultry, that causes both subclinical and clinical forms of the disease (Prescott et al., 2016). Necrotic enteritis has a variety of factors that pre-dispose a flock to experiencing an outbreak. These include, but are not limited to, the ingredients used in the feed, immunosuppression, and coccidia infections.

In the past, NE was well controlled using subtherapeutic levels of antibiotics in the feed. Now, access to these control measures has become restricted, mainly due to consumer pressure for antibiotic free production and the release of the Veterinary Feed Directive in response to the concerning rise in antibiotic resistant genes (FDA, 2019). As a result, the incidence of NE has become much higher in production flocks of broilers and often leaves producers with limited options to treat it. Birds can be treated by a vet with strong antibiotics, but this becomes expensive, limits food chain options, and there is no guarantee they will recover, especially if faced with antibiotic resistant strains of *C. perfringens*. Probiotics and direct fed microbials (DFM) have become popular products for control of NE, likely because they are easy to

administer, federal regulations are minimal, and costs are low. Probiotics and DFM's can be selected for specific bactericidal effects, especially *Bacillus* species that are notorious enzyme producers. *Bacillus*, with few exceptions, do not pose a disease risk and their spore-forming ability makes them appealing for inclusion in feed, which makes administration easy and consistent for poultry producers. However, it is important that these DFM's have activity within the targeted disease area of the gut, and *in vitro* tests can offer insight into the probability that a candidate DFM will be successful.

Chapter 2.

Review of the Literature

Necrotic Enteritis

As of 2015, clinical and subclinical necrotic enteritis (NE) had an estimated global economic impact of about $6 billion annually (Wade and Keyburn, 2015). Necrotic enteritis is a short-term disease that impacts individual birds for a few days but may roll through a flock more chronically. While NE has been historically controlled fairly well by antibiotics provided at sub-therapeutic and therapeutic levels, access to these control measures has become restricted, mainly due to consumer pressure and the Veterinary Feed Directive (FDA, 2019). This has resulted in increased pressure to control the disease through non-antibiotic methods

Necrotic enteritis is a form of enterotoxaemia primarily caused by *Clostridium perfringens*, an opportunistic, anaerobic, spore-forming bacteria, normally residing in the gastrointestinal tract (GIT) of poultry, that causes both subclinical and clinical forms of the disease (Prescott et al., 2016). Clinical NE affects broilers between 2-5 weeks of age and has a rapid onset, with the primary symptoms being severe depression and diarrhea, followed by a significant spike in mortality. The easiest method of diagnosis is by observing the small intestines of birds who have succumbed to illness. During a necropsy, clinical signs include ballooned and fragile small intestines, a rancid smell, sloughing mucosal layers, and the diphtheritic-like appearance of the small intestine as the mucosal epithelium sloughs (Hargis,2014).

According to Olkowski et al. (2008), when looking at the damage caused by NE to the GIT, there are three distinct phases in the progression of tissue necrosis and lesion formation.

First, edema accumulates between the lamina propria and the enterocytes. This causes a histologically observable separation of the epithelium from the basement membrane while few cells show signs of actual necrosis. The cellular disruption then continues with the progressive destruction of the lamina propria and signs of necrosis beginning to appear among enterocytes. The final step of microscopic lesion formation is the total loss of all structural features of the lamina propria as well as all signs of necrosis progressing in the enterocytes leading to total disruption of the mucosal and epithelial lining of the GIT.

One of the most important aspects when evaluating an NE outbreak is the presence of predisposing factors. Some of the most common factors within a flock are feed composition, birds' immune status, and existing intestinal damage (Riddell and Kong, 1992; Yegani and Korver, 2008; Rodgers et al., 2014). Certain ingredients, such as poorly digestible protein sources, create an over availability of protein in the lumen of the small intestines that *C. perfringens* uses as substrate causing overgrowth within the GIT. Alternatively, grains like wheat, oat, and barley that are high in non-starch polysaccharides can increase digesta viscosity, slowing feed passage rate and providing substrate for *C. perfringens* sporulation and growth (M'Sadeq et al., 2015). Immunosuppression can be caused by a variety of environmental and biological factors making it a devastating result of poor management. Factors such as overcrowding or high levels of ammonia can cause immunosuppression (Hoerr, 2010). High environmental ammonia levels have been shown to reduce size of spleen, thymus, and bursa of Fabricius, which are all integral immune organs for poultry, as well as reduced blood lymphocyte concentration and increase gene expression for a variety of interleukins (Wei, 2015). Infection with bacteria such as *Salmonella enterica* serovar Typhimurium or viral diseases such as Marek's, infectious bursal disease, or chicken anemia virus also create a compromised immune

status, giving *C. perfringens* the perfect foot hold to cause NE (Moore, 2016). The third, and most common, predisposing factor of NE is existing damage to the lining of the intestinal wall, especially that caused during a coccidia infection. *Eimeria* oocysts, either from a wild-type infection or during over-cycling of a vaccine, colonize the intestinal wall and kill epithelial cells causing plasma leakage into the intestinal lumen that will be used as a substrate by *C. perfringens* for over proliferation.

Predisposing Factors

Salmonella Typhimurium

Salmonellae are Gram-negative, rod shaped, facultative anaerobic bacteria belonging to the family Enterobacteriaceae. *Salmonella* Typhimurium is a common, antibiotic resistant, nontyphoidal serovar in poultry and is noted for its pathogenicity and ability to cross-infect humans when it enters the food chain (Andino and Hanning, 2015). *Salmonella* Typhimurium has been established as a potent predisposing factor of NE when administered on day-of-hatch to broiler chicks (Shivaramaiah et al., 2011). Infection on day-of-hatch with *Salmonella* Typhimurium produces a significant decrease in body weight, body weight gain, and feed intake in as little as seven days (Latorre et al., 2018). Along with the decrease in overall bird performance, infection with *Salmonella* Typhimurium on day-of-hatch is linked to persistent intestinal inflammation and promotion of *C. perfringens* colonization and pathogenesis due to early intestinal lesion formation (Beal et al., 2004). *Salmonella* Typhimurium infection has also long been established as deteriorating the immunocompetence of birds that have been inoculated on day-of-hatch (Hassan and Curtiss, 1994). The combination of lymphocyte depletion, atrophy

of immune organs, and reduction of lymphatic follicular formation creates an immunocompromised state in birds, giving rise to a more ideal environment for *C. perfringens* overgrowth and an outbreak of necrotic enteritis (Hassan and Curtiss, 1994).

Coccidiosis

As mentioned earlier, the predominant predisposing factor of clinical necrotic enteritis is coccidia infection with *Eimeria* spp. *Eimeria* are a family of intracellular protozoal parasites that invade the epithelial lining of GIT in birds and destroy host epithelial cells, causing significant damage to the intestinal wall (Quiroz-Castañeda and Dantán-González, 2015). This damage causes leakage of plasma into the intestines, which is then available as a protein rich substrate for the growth of *C. perfringens* (Rodgers et al., 2014). This, paired with increased mucin production mounted by the inflammatory response, allows for over colonization of *C. perfringens* and the very high rate of bird mortality commonly associated with necrotic enteritis (Timbermont et al., 2011).

Coccidia infections are very difficult to get rid of once they have taken hold in a flock. Between the short replication period of 4-6 days during the endogenous stage and the hard, protective coating of sporulated oocysts during the exogenous stage, *Eimeria* have evolved as very effective parasites that can persist for generations in flocks if not kept in check (Chapman, 2014). While the best way to control *Eimeria* is through proper husbandry and biosecurity, outbreaks can still occur. In the event of an outbreak, most producers will use a rotational schedule of anticoccidial ionophore administration to kill off the parasites while minimizing the rise of emergence of drug resistant strains (Peek and Landman, 2011).

While ionophores are very good at controlling coccidiosis, their use in controlling necrotic enteritis is situational at best and does not eliminate the risk of necrotic enteritis lesions forming. Anticoccidial ionophores such as lasalocid, salinomycin, and monensin do have observable antibacterial effects against *C. perfringens*, according to Martel et al. (2004), but they are not nearly as effective against bacteria as traditional antibiotics, and their control of necrotic enteritis is primarily through a reduction of GIT damage caused by *Eimeria*. Additionally, when looking at outbreaks of necrotic enteritis, ionophores are most effective against infections that are caused by coccidiosis as the predisposing factor, as their primary purpose is to reduce the load of coccidia within the GIT. Anticoccidial ionophores also cannot be used in flocks marketed as "no antibiotics ever" or "raised without antibiotics", further limiting the practicality of using them as a means of control for necrotic enteritis (Fancher et al., 2020).

Clostridium perfringens

C. perfringens is a Gram-positive, anaerobic bacterium that is ubiquitous in the environment and within poultry populations. Since *C. perfringens* is ubiquitous and a common resident within the small intestines, it is not possible to just remove the bacteria as a means of preventing NE, so most strategies focus on controlling the clostridial load either in the GIT or the environment. Another key factor is that not all strains of *C. perfringens* can cause an outbreak of NE. At this time, only those strains capable of producing alpha toxin, NetB toxin, or both are considered candidates for causing NE and only in the presence of preexisting conditions, such as those described above.

C. perfringens can produce a wide variety of antigens that can cause damage to a host if not kept in check. These include the "major" toxins *alpha, beta, epsilon, iota,* and enterotoxins, as well as the "minor" toxins *gamma, eta, delta, theta, kappa* (collagenases), *lambda* (protease), *nu* (deoxyribonuclease), and *mu* (hyaluronidase) toxins (McDonel, 1980). Based on the ability of a strain to produce one or more of the four major toxins, alpha toxin (CPA), beta toxin (CPB), epsilon toxin (ETX), and iota toxin (ITX), it is placed into one of five categories, known as toxinotypes. These toxinotypes are labeled as type A, which only produce CPA, type B, which produce CPA, CPB, and ETX, type C, which produce CPA and CPB, type D, which produce CPA and ETX, and type E, which produce CPA and ITX (Uzal et al., 2010). The toxinotyping system for the types of *C. perfringens* has recently been updated by Mehdizadeh-Gohari et al. (2021) from a five point to a seven-point system, labeled types A-G. The seven types are distinguished by the presence or absence of six different types of toxins instead of just the original four: alpha toxin (CPA), beta toxin (CPB), epsilon toxin (ETX), iota toxin (ITX), *C. perfringens* enterotoxin (CPE), and necrotic enteritis B-like toxin (NetB). While this classification is very new, and not yet widely accepted, it is a much more accurate representation of the pathogenicity of *C. perfringens* and offers more information about a strain's pathogenic potential.

As defined by Mehdizadeh-Gohari et al. (2021), with input on the effect toxins have and the diseases they can cause from Petit et al. (1999), type A species of *C. perfringens* are denoted as only having the ability to produce CPA. Type A strains can cause gas gangrene in humans and animals, enterotoxaemia and GI disease in ruminants, hemorrhagic gastroenteritis in dogs and horses, and may play a role necrotic enteritis in birds. Type B strains produce CPA, CPB, and ETX. The only disease type B strains are known to cause is lamb dysentery. Type C strains are

known to release CPA and CPB and have the potential to produce CPE based on acquired plasmids. Type C strains are known to cause a variety of GI issues such as hemorrhagic and necrotizing enteritis in neonatal animals. Type D strains produce CPA and ETX and have the potential to produced CPE based on acquired plasmids. In ruminants, type D strains can cause enterotoxaemia if *C. perfringens* colonies are given the chance to proliferate. Type E strains produce CPA and ITX and have the potential to produced CPE based on acquired plasmids. The pathogenicity of type E strains is still debated, but there is some evidence supporting that it can cause gastroenteritis in cattle and rabbits. Type F strains are capable of releasing CPA and CPE and are a significant contributor to human food poisoning and bacterially derived diarrhea. The final classification, type G strains, is known to produce CPA and NetB. These types of strains, denoted as type A strains by the old toxinotyping system, are noted as being the primary causes of necrotic enteritis in poultry and are being heavily researched to find ways to stifle their growth due to the impact of necrotic enteritis on broiler production.

Clostridium perfringens **toxins**

When necrotic enteritis was first characterized in chickens, it was determined by Al-Sheikhly and Truscott (1977) that the primary virulence factor for causing the disease was the presence of alpha toxins produced by type A strains of *C. perfringens.* Alpha toxin is a two-domain zinc metalloenzyme that is resistant to proteases and can bind to host cell membranes when calcium ions are present. The two domains are designated as the C-domain and the N-domain. The C-domain is very similar in structure to the C2-like domains of eukaryotic proteins involved in signal transduction (Sakurai et al., 2004). Based on this similarity in structure, it is inferred that the C-domain is primarily used by the toxin to bind to the phospholipid bilayer of

the cell membrane. The N-domain is similar in topology to *Bacillus cereus* phospholipase C. Following binding of the C-domain to the cell membrane, the N-domain hydrolyzes either phosphatidylcholine or sphingomyelin (Jewell et al., 2015). Based on their binding sites and substrates, the C-domain is primarily used by the toxin to bind to the membrane of a cell while the N-domain is responsible for hydrolyzing the membrane and causing cell damage or death.

When studying damage caused by alpha toxin, it is important to account for toxin concentration as this will impact the extent of tissue damage caused, as well as what pathways are activated following damage. In high concentrations, alpha toxin causes massive degradation of phosphatidylcholine and sphingomyelin in membranes leading to obvious cell death and tissue necrosis, giving rise to lesions that are indicative of a necrotic enteritis infection. However, in low concentrations, alpha toxin only causes limited hydrolysis of phosphatidylcholine and sphingomyelin but will cause a variety of symptoms depending on the infected tissue, such as cascades that mimic endogenous phospholipase C activity (Meyers and Berk, 1990). In low concentrations, alpha toxin causes a variety of secondary symptoms that result in further tissue damage such as mis-trafficking of neutrophils, vasoconstriction and platelet aggregation, and activation of the arachidonic acid cascade and protein kinase C (Titball et al., 1999).

Many host factors are interfered with by the alpha toxin, causing secondary tissue damage and further complications from disease. The first pathway affected is immune cell signaling and trafficking where the interference by alpha toxin causes neutrophils to accumulate in blood vessels surrounding infected tissue and prevents them from entering tissue due to binding of adhesion molecules (Stevens et al., 1997). Alpha toxin then causes activation of the arachidonic acid cascade which leads to production of products such as thromboxanes,

leukotrienes, and prostaglandins that facilitate a localized inflammatory response and promote vasoconstriction, causing anoxia in affected tissues (Fujii and Sakurai, 1989). Finally, the production of diacylglycerol and protein kinase C during cell membrane damage causes a massive signaling cascade, inducing further damage to the cell membrane by activating endogenous phospholipases in the host and causing platelet aggregation to further reduce blood flow to the infected area (Whatley et al., 1989; Kald et al., 1994). The combination of these host factors further contributes to cell death, and in the case of necrotic enteritis, lesion formation and tissue necrosis.

Historically, it was thought that only strains capable of producing alpha toxins were the causative agent of NE (Al-Sheikhly and Truscott, 1977a; b). Recently however, a new virulence factor, NetB toxin, has also been shown to induce NE outbreaks, some even in the absence of genes for production of alpha toxin (Keyburn et al., 2008). NetB toxins are unlike most others associated with *C. perfringens* and show more similarities with alpha toxins produced by *Staphylococcus aureus*. The NetB toxin works by binding to receptors on the membrane surface of a cell, followed by oligomerization and pore formation (Islam et al., 2021).

NetB is a beta barrel, pore forming toxin belonging to the hemolysin family that shows a high affinity, for avian-derived cells, although the reason behind this is not yet fully understood. It is produced as a soluble precursor that then binds to host's cell membranes, assembles itself as an oligomer, and forms a transmembrane pore (Yan et al., 2013). The formed pore has a heptameric structure that contains bilayer spanning, antiparallel beta barrels very similar in structure to those of alpha toxins produced by *Staphylococcus aureus* (Song et al., 1996). The pores that NetB toxin forms tend to remain open and show preference to cations over anions

(Shatursky et al., 2000). This cationic preference causes an influx of sodium, potassium, and calcium ions followed by an influx of water leading to osmotic cell lysis (Rood et al., 2016). The influx of ions induces rounding and lysis of cells, followed by a release of lactate dehydrogenase (LDH) which can be used as a marker for cell damage and necrosis (Chan et al., 2013). In general, NetB toxin causes tissue necrosis through destruction of the lamina propria and the formation of pores in the extracellular matrix and intercellular junctions, causing observable lesions within the GIT (Olkowski et al., 2008).

Necrotic Enteritis Control

In the past, necrotic enteritis was well controlled by subtherapeutic antibiotics in feed, but since they have been phased out of use, necrotic enteritis has become a much more prevalent issue in broiler production. Traditionally, antibiotics such as avilamycin, avoparcin, and bacitracin were used for the control of necrotic enteritis (Prescott et al., 1978; Elwinger et al., 1998), before fears of antimicrobial resistance genes arose (Armbruster and Roberts, 2018). As a result of their prolific success in the past, antibiotics such as amoxicillin, tylosin, and lincomycin (Lanckriet et al., 2010) are still used as a gold standard for which to test alternative methods of prevention against as, assuming the absence of resistance genes, these antibiotics prevent the development of any necrotic lesions in birds even in the presence of predisposing factors (Hermans and Morgan, 2007). As the poultry industry has moved away from the use antibiotics for prophylactic and therapeutic treatment of necrotic enteritis, the challenge has been presented to find alternative means of treatment and prevention. Among the potential strategies for combating necrotic enteritis, there is the use vaccines against toxins or conserved genes of *C.*

perfringens to prime the immune system against an outbreak of necrotic enteritis and the use of feed additives like pre- and probiotics to modulate the microflora within the GIT.

Vaccination against *C. perfringens* has received increased attention in research recently as a potential means of controlling overgrowth and preventing necrotic enteritis. When devising a vaccine, it is important to ensure that the antigens used are specific enough to *C. perfringens* that other potentially beneficial bacteria will not be recognized by the immune system, but not too specific to offer as much coverage of the species *C. perfringens* instead of just a specific strain or toxinotype. Proper vaccine development hinges on the ability to locate peptides that are highly conserved among a species of bacteria, that do not interfere with host pathways or beneficial bacterial processes, and will produce antigens in high enough quantities to trigger an immune response (Duff et al., 2019). The specificity required to develop an effective vaccine is what makes them so effective but is also the reason an all-encompassing vaccine has not been produced yet. While vaccines work well, when properly developed, at priming the immune system and preventing outbreaks of disease, it is very difficult to generate one that would offer the coverage required to control such a ubiquitous and varied pathogen as *C. perfringens.*

When evaluating feed additives for the prevention of NE, the focus is generally divided between two main groups: prebiotics, which have been defined as “substrates, that are selectively utilized by host microorganisms, conferring health benefits” by the International Scientific Association for Probiotics and Prebiotics, or probiotics, also known as direct fed microbials (DFM), which, as defined by the United States Food and Drug Administration, are products “purported to contain live microorganisms”.

Prebiotics vary widely in their composition, ranging from peptide chains to natural products such as essential oils and herbs, and even can be comprised of organic or synthetic bactericides aimed at targeting potential pathogens within the GIT (Roberfroid et al., 2010). They also vary greatly in their target microbe, as, in theory, any normal resident of the GIT could become a potential target for a selected prebiotic additive. Due to their variety in composition and target, the effectiveness of prebiotics is highly variable, as there is no way to ensure these additives make their way in a high enough concentration to their specific target or that their target has a strong enough presence in the GIT to see a benefit to the use of a particular prebiotic. Due to the variability of effectiveness of prebiotics, probiotics have been heavily investigated, especially with regards to inhibiting the growth of *C. perfringens* to prevent and control outbreaks of necrotic enteritis (Fuller, 2012). As described by Paiva and McElroy (2014), probiotics contain known beneficial bacteria that aim to alter the host's microbial population in some way and can do so via several modes of action. The first is by maintaining the host's native microbial population via competitive exclusion against pathogenic microbes. The next mode of action is by altering the host's enzymatic activity and ammonia production while improving feed intake. The final is that probiotics are capable of neutralizing enterotoxins, producing natural antibiotics, and stimulating the immune system to fight off pathogens (Dahiya et al., 2006).

Probiotics of Interest

Lactic acid producing bacteria (LAB) have a wide range of beneficial effects as probiotics and are currently in use, for both humans and animals, for disease prevention and maintenance of the gut microflora (Tavakoli et al., 2017). One genus of beneficial LAB probiotics is *Lactobacillus*, which is a large group of rod-shaped, Gram-positive, non-spore

forming bacteria that are currently used as a feed supplement to reduce the incidence of intestinal diseases (Higgins et al., 2008) as well as to maintain a healthy microbiota in poultry (Riaz-Rajoka et al., 2017). Lactobacilli also produce anti-inflammatory and bacteriostatic products (Schreiber et al., 2009) that help to modulate immune responses, namely by accelerating phagocytosis processes in macrophages (Brisbin et al., 2015). Through these processes, Rajput et al. (2017) has shown LAB probiotics cause a beneficial interaction with the host to combat pathogens such as *Salmonella*, *E. coli,* and *C. perfringens.*

Another LAB probiotic that is a normal member of human and animal microbiomes is *Enterococcus faecium,* a Gram-positive, cocci shaped, facultative anaerobe. This probiotic produces enterocins and organic acids with antimicrobial properties that combat *C. perfringens* growth (Klose et al., 2010). Along with its antimicrobial properties, *E. faecium* has been shown by Samli et al. (2006) to increase villus surface area of the host, aiding nutrient absorption and improving weight gain. *E. faecium* was also shown to modulate the intestinal microflora and stimulate the immune response throughout the GIT by Rajput et al. (2017), increasing the host's ability to prevent infections from pathogens such as *Salmonella*, *E. coli,* and *C. perfringens.*

Bacillus is a genus of Gram positive, rod-shaped bacteria with a very large number of named species of bacteria belonging to it. *Bacillus* species have several advantages when applied as a probiotic such as preexistence in the feed manufacturing process and increased shelf life of feed when used as an additive (Grant et al., 2018). Additionally, *Bacilli* can form endospores which enhances their ability to survive harsh environments such as the high heat pelleting process of feed or the fluctuating pH extremes and hydrolytic enzymes of the GIT (Elshaghabee et al., 2017). Many *Bacillus* species are considered good candidates for probiotics as they already

exist in varying capacities within the host microbiota and produce beneficial products to the host such as anti-inflammatories and bacteriocins that can have activity against undesirable bacteria (Eichner et al., 2018). *Bacillus* bacteria, specifically, are investigated as probiotics for using in controlling necrotic enteritis outbreaks due to the fact that they are prolific producers of bacteriocins that have negative effects on the growth of *C. perfringens* (Caly et al., 2015). These bacteria have evolved to produce a wide variety of enzymes, some of which are bacteriocins, with effects such as reducing *C. perfringens* growth, either by killing live cells or preventing cellular adhesion to the intestinal lining, as well as neutralizing the toxic effects of various strains of *C. perfringens* through a variety of methos including inhibition of genes for toxin production (Kawarizadeh et al., 2019). The *Bacillus* genus is also shown to promote intestinal development, measured as height of intestinal villi, thus improving the surface area for absorption of nutrients (Pluske et al., 1996), produce a variety of hydrolytic enzymes that aid in feed break down and enhance absorption further (Rozs et al., 2001), and form a protective barrier along the wall of the intestines that interferes with pathogen attachment and further neutralizes toxins (Rajput et al., 2013).

Conclusion

Necrotic enteritis is a disease of increasing importance within the poultry industry responsible for billions of dollars in losses around the world. NE is caused by *Clostridium perfringens* and relies on other predisposing factors to cause an outbreak. These predisposing factors most commonly include immunosuppression, such as is seen in an infection with *Salmonella* Typhimurium, and existing damage to the lining of the intestines such as that commonly caused by coccidia infections. Due to pressure from consumers and the VFD, in feed

antibiotics are no longer a viable option for prevention and control of NE outbreaks. As a result, alternative means are being investigated as potential solutions to control and treat NE. Due to the ever-growing knowledge bank of information surrounding the pathogenicity of *Clostridium perfringens* and the lack of understanding surrounding the more novel toxins and their roles as virulence factors, targeted prevention methods, such as vaccination, are difficult to develop making broad spectrum preventatives a more promising area of study for the time being. One of the most promising areas widely investigated is the use of direct fed microbials, or probiotics, that exhibit anti-clostridial properties to modulate the microbiota of poultry and prevent the overgrowth of CP, even in the presence of the predisposing factors described above. *Bacilli*, as a genus, are robust bacteria capable of surviving varied environments while producing bacteriocins that can modulate the microbiota and control growth of opportunistic pathogens such as CP. Further investigation must be performed to determine which strains of *Bacilli* would act as the most promising probiotic for use as a feed additive to control outbreaks of NE. Additionally, further research should be preformed into the composition of the antimicrobials produced by probiotics as this could be a means for developing new antimicrobial products to combat pathogens that exhibit high incidences of antimicrobial resistance and can give a basis for the structure or new potential modes of action of new naturally and artificially produced antimicrobials.

Chapter 3.

Characterization of the anti-clostridial effects and survivability of the novel probiotic

Abstract

Necrotic enteritis is an enteric disease primarily caused by overgrowth of *C. perfringens* (CP) in the small intestine following a variety of predisposing factors. The objective of this study was to determine if a novel probiotic showed anti-clostridial effects, survived pelleting temperatures and the varied environment of the gastrointestinal tract (GIT), and if anti-clostridial effects were retained through the GIT. The probiotic was tested against 9 strains of CP to determine overarching anti-clostridial effect. The probiotic suppressed all 9 strains of CP significantly (p-value<0.05) when CP inoculated media was overlayed onto a pre-grown colony of probiotic and zones of inhibition measured. Next, probiotic efficacy was compared against common antibiotics and commercial probiotics. Two of the four antibiotic treatments, penicillin (0.0625mg/mL) and metronidazole (0.05mg/mL), both commercially available probiotics, and the candidate probiotic were all able to reduce CP growth, with the candidate probiotic outperforming both commercial probiotics (p-value<0.001) and metronidazole (p-value$=0.007$). The CP strain showed resistance to the other two antibiotic treatments, BMD (0.022mg/mL) and avilamycin (0.05mg/mL).

A germination and sporulation assay was completed to ensure spores could germinate at the internal body temperature of chickens and sporulate to survive pelleting. A lack of significant change (p-value$=0.096$) in cell recovery was indicative of the probiotic's ability to germinate to colonize the gut and sporulate to endure pelleting. Interaction of the probiotic candidate with 18 other common enteric residents of the microbiome in poultry were observed in a streak plate

assay, in search of potential inhibition. Of the 18 additional strains tested against the probiotic, three strains of *E. cecorum* were significantly inhibited by the probiotic based on Chi^2 analysis. A simulation digestive assay was performed mimicking the crop, proventriculus, and intestines to ensure the probiotic could survive the gastrointestinal tract. When spores recovered from each section of the GIT were compared, the final concentration was statistically lower than the initial (p-value=0.018) with a 0.35-Log_{10}-fold reduction in the recovered CFU reaching the small intestine. The survival assay was repeated with the addition of 10^8 CFU of CP strain TXAM 020410 in the small intestines portion of the assay to determine if the efficacy of the anti-clostridial properties was maintained. Using a 10^6 dose of spores, the reduction in CP was approaching significance at p-value=0.112 but saw a reduction of nearly 1-Log_{10} CFU of recovered CP. The results of these experiments indicate the probiotic is a candidate for treatment and control of necrotic enteritis due to its broad anti-clostridial properties and resilience in harsh environments.

Materials and Methods

Overview of Experimental Design

The anti-clostridial effects, survivability, and overall potential of the novel probiotic candidate were characterized through a series of six *in vitro* experiments (3.1, 3.2, 3.3, 3.4, 3.5, and 3.6). Experiment 3.1 used the probiotic in an overlay inhibition assay to determine the broad-spectrum antimicrobial effects against several strains of *C. perfringens*. Experiment 3.2 used an overlay inhibition assay to compare the anti-clostridial effects of the novel probiotic against some commonly used antibiotics and commercially available probiotics already in use for necrotic enteritis prevention. Experiment 3.3 tested the ability of the probiotic to germinate and sporulate as necessary, depending on the temperature of the environment it encountered. Experiment 3.4 used a streak plate method to determine if any inhibition occurred when the probiotic interacted with other potential opportunistic pathogens or probiotics. Experiment 3.5 involved the use of a digestive simulation assay to mimic the various conditions of the crop, proventriculus, and small intestines of a chicken to ensure the probiotic could reach its desired site of colonization in a high enough quantity to colonize the small intestines. Finally, experiment 3.6 used the digestive simulation assay to ensure that the anti-clostridial effects of the probiotic were maintained as it traveled through the digestive tract.

Experiment 3.1: Anti-clostridial activity

To characterize the spectrum of anti-clostridial activity of the *B. velezensis* probiotic, a soft agar overlay inhibition assay was performed using the candidate probiotic against nine pathogenic strains of *C. perfringens*. For the assay, *B. velezensis* spores were suspended in saline

and a 10 µL drop was plated on the center of an LB agar plate. The LB plate was incubated aerobically overnight at 37°C to allow the probiotic to grow in a pellet in the center of the plate (Mundo et al., 2004). While the probiotic is grown on the plate, the nine strains of *C. perfringens* (641, ATCC 10543, ATCC 13124, BB, CP 1, CP 2, Todd, TXAM 011610, and TXAM 020410) were grown in TSB + 0.05% Thioglycolate anaerobically, on a shaker, at 37°C until turbid. The following day, 15 mL of melted TSB-based soft overlay agar, formulated to promote *C. perfringens* growth, was inoculated with 1 mL of a single strain of *C. perfringens* and poured over the pre-grown colony of the candidate probiotic. The soft agar was allowed to solidify and was then incubated anaerobically, overnight, at 37°C. After the final incubation, the plates were removed from the incubator, checked for a zone of inhibition, and, if one was present, were measured (in mm) in three places, from the edge of the probiotic colony to the outer edge of the zone of inhibition.

Experiment 3.2: Anti-clostridial efficacy

To get an idea of how efficiently the probiotic candidate was able to inhibit the growth of *Clostridium perfringens*, a simulation of an inhibition sensitivity test was performed using a method similar to the agar overlay inhibition assay from experiment 3.1. Eight treatments were established, 4 bacterial treatments (the novel *B. velezensis* probiotic, a commercially available B11, a GMO *B. subtilis* strain, and a strain of *E. coli*) and 4 antibiotic treatments (penicillin (0.0625 mg/mL), metronidazole (0.05 mg/mL), BMD (0.022 mg/mL), and avilamycin (0.010mg/mL)). For the bacterial treatments, a 10 µL drop of bacteria was plated on the center of an LB agar plate. The LB plate was incubated aerobically overnight at 37°C to allow the probiotics to grow in a pellet in the center of the plate. While the bacterial treatments were

growing on the plates, the selected strain of *C. perfringens* (strain TXAM 020410) was grown in TSB + 0.05% Thioglycolate anaerobically, on a shaker, at 37°C. The following day, the 4 antibiotic treatments were prepared by dipping a piece of sterile filter paper into a solution containing each antibiotic at a known concentration. The filter paper had excess liquid knocked off and was placed in the center of an LB agar plate to dry. After the 8 treatments had been prepared, 15 mL of melted TSB-based soft overlay agar, formulated to promote *C. perfringens* growth, was inoculated with 1 mL of *C. perfringens* strain TXAM 020410 and poured over the pre-grown bacterial colonies or antibiotic treatments. The soft agar was allowed to solidify and was then incubated anaerobically, overnight, at 37°C. After this, the plates were removed from the incubator, checked for a zone of inhibition, and, if one was present, were measured (in mm) in three places, from the edge of the probiotic colony to the outer edge of the zone of inhibition.

Experiment 3.3: Germination and Sporulation

Since the probiotic will be delivered to the birds in a sporulated form in feed, an experiment was developed to ensure that the spores would survive at the internal body temperature of a chicken and through the high heat process of pelleting at the OSU feed mill. To simulate these conditions, 1.25g of sterile feed and 10^7 spores were suspended in 4.5 mL of a sterile 0.9% saline + 0.1% Tween + 10mmol EDTA solution. The solution was thoroughly mixed, and then placed in a water bath at 40°C for 10 minutes to simulate the internal body temperature of a chicken. After this 10-minute incubation period was up, a subsample was removed from each replicate, for CFU enumeration, and then the tubes were placed in a separate water bath at 75°C for an additional 10 minutes, to remove vegetative cells so only spores that

survived the high temperature would be measured. After this final incubation, a subsample of each replicate was removed for CFU enumeration.

Experiment 3.4: Effect of B. velezensis on other members of the microbiota

To get an idea of how the novel *B. velezensis* probiotic interacted with some other common residents of the poultry microbiota, a streak plate inhibition assay was developed and performed using 18 strains of bacteria commonly used as probiotics in poultry or that can act as common opportunistic pathogens. Of the 18 strains used to test against the *B. velezensis* probiotic candidate, 7 were strains of *Enterococcus cecorum,* 4 were strains of *Escherichia coli,* 4 were strains of *Salmonella enterica,* 1 was a *Bacillus subtilis,* 1 was a *Lactobacillus,* and 1 was a *Pediococcus.* To prepare this assay, the probiotic candidate, as well as the 18 strains of bacteria to be used against it, were pre-grown according to their respective growth conditions. A sterile swab was then dipped into the *B. velezensis* and dragged across the length of an LB agar plate down its center and allowed to dry. A different sterile swab was then dipped into a single strain of test bacteria and dragged across the dried streak of *B. velezensis*. Ten replicates of each strain were plated across the *B. velezensis* for a sample size of n=10. Three strains were streaked across the *B. velezensis* on each plate, allowed to dry, and incubated overnight at 37°C according to the growth conditions of each strain. The following day, plates were observed for visible signs of inhibition to characterize the interactions *B. velezensis* had with common residents of the poultry microbiota.

Experiment 3.5: Gastrointestinal Survival Assay

Since the novel probiotic will be delivered to birds in feed as a DFM, it must be able to travel through most of the gastrointestinal tract to reach the small intestines, the desired region of antimicrobial activity. To assess how traveling through the gut affects spore concentrations, a simulation digestive assay was developed to mimic the conditions of the crop, proventriculus, and small intestines of chickens. These studies were conducted according to Lattore et al. (2018). To simulate these conditions, 5g of sterile feed and 10^6 spores of the probiotic were suspended in 10 mL of 0.03M HCl, and the pH was adjusted to about 5.2. The tubes were then incubated at 40°C on a shaker for 30 minutes to simulate crop digestion. The tubes then had their pH adjusted to between 1.4 and 2.0 using 1N HCl and had 15,000 U of pepsin added to each. The tubes were again incubated at 40°C on a shaker for an additional 45 minutes to mimic the digestive conditions of the proventriculus. The final step was to adjust the pH of the tubes once again to be between 6.4 and 6.8 using 1M $NaHCO_3$ while adding 6.84 mg of 8x pancreatin to each tube and incubate again at 40°C on a shaker for 120 minutes to mimic the conditions of the small intestines. At the start of the assay, and after every subsequent incubation period, a subsample was removed from each tube for enumeration of CFU.

Experiment 3.6: Gastrointestinal Efficacy Assay

The final experiment of the *in vitro* portion of this study was to ensure that the anti-clostridial properties of the novel probiotic candidate, exhibited in the previous experiments, were maintained as the probiotic was exposed to the varied environment of the gastrointestinal tract. To do this, the simulation assay from experiment 3.5 was repeated with some changes. 5g of sterile feed and 10^6 spores of the probiotic were suspended in 10 mL of 0.03M HCl, and the pH was adjusted to about 5.2. The tubes were then incubated at 40°C on a shaker for 30 minutes

to simulate crop digestion. The tubes then had their pH adjusted to between 1.4 and 2.0 using 1N HCl and had 15,000 U of pepsin added to each. The tubes were again incubated at 40°C on a shaker for an additional 45 minutes to mimic the digestive conditions of the proventriculus. The final step was to adjust the pH of the tubes once again to be between 6.4 and 6.8 using 1M $NaHCO_3$ while adding 6.84 mg of 8x pancreatin to each tube to mimic the conditions experienced during intestinal digestion. Then 10^8 CFU of *C. perfringens* strain TXAM 020410 were added to each tube along with sodium thioglycolate to act as an oxygen quencher in the solution. The tubes were placed under anaerobic conditions and incubated again at 40°C on a shaker for 120 minutes to mimic the small intestines. A subsample was removed after the final incubation for *C. perfringens* CFU enumeration.

Statistical Analysis

Analysis on the zones of inhibition (mm) and the changes in Log_{10} CFU concentration of bacteria were analyzed using a one-way ANOVA in JMP Pro 14 statistics using the Tukey-Kramer method for the comparison of means with a statistical significance set to $p<0.05$. The streak plate comparison for the effect of *B. velezensis* on other microbiota were analyzed in Excel using a Chi squared analysis with a significance value set at 3.841 based on the number of replicates per treatment (n=10).

Results and Discussion

Experiment 3.1: Anti-clostridial activity

To establish the extent of broad spectrum anti-clostridial activity, the novel *B. velezensis* probiotic was tested against nine strains of *C. perfringens* in an overlay inhibition assay. As shown in **Table 1,** the novel probiotic was capable of significantly inhibiting the growth of all nine strains of *C. perfringens* (p-value<0.01). The variation observed in the radial size of the zones of inhibition (mm) generated between strains ranged from just over 7mm in the case of strain TXAM 011610 to just under 16mm against strain CP2. This is more than likely due to the inherent variation between the strains as they express different antimicrobial resistance genes as well as the difference in their growth rate. Based on how successful the novel probiotic was at inhibiting all nine strains of *C. perfringens* tested against it, the broad spectrum anti-clostridial properties of the *B. velezensis* strain are inherently potent and not inhibited by differences in gene expression between strains of *C. perfringens.*

Experiment 3.2: Anti-clostridial efficacy

After displaying the broad spectrum anti-clostridial activity of the novel probiotic, the agar overlay inhibition assay was repeated with a variety of different treatments to determine how effective the *B. velezensis* probiotic was at inhibiting clostridial growth compared to some common preventatives and control measures. Eight treatments were established, four antibiotic and four bacterial, to be used against *C. perfringens* strain TXAM 020410. TXAM 020410 was chosen as the test strain as it is a heartier, faster growing strain of *C. perfringens* that is known to exhibit a variety of antimicrobial resistance genes, as can be seen in **Table 2**. Based on the zones

of inhibition that were generated, two of the four antibiotics, penicillin (14.37±0.58 mm) and metronidazole (6.97±0.34 mm), were able to inhibit the growth TXAM 020410 significantly (p-value<0.01) with penicillin being the most effective treatment administered overall. The other two antibiotics, BMD (0±0 mm) and avilamycin (0±0 mm), were incapable of inhibiting clostridial growth. Of the bacterial treatments, the novel *B. velezensis* (8.36±0.2 mm) was the most successful, based on the size of zone of inhibition generated. The *B. subtilis* strain was also able to inhibit the growth of *C. perfringens* significantly (6.31±0.083 mm), while the commercially available probiotic, B11, had a less significant effect (2.25±0.072 mm). The final bacterial treatment used was a strain of *E. coli* which was not known to exhibit any anti-clostridial properties. Based on the zone of inhibition generated by this treatment (1.46±0.11 mm), an idea of how resource utilization would affect clostridial growth was able to be developed and give merit to the conclusion that TXAM 020410 was also resistant to the commercially available B11 probiotic. Significance of the zones of inhibition was determined by comparison with plates that had received no treatment as a control.

Experiment 3.3: Germination and Sporulation

Based on the lack of a significant decrease, as seen in **Table 3**, in the recovery of spores from the start of the assay to the end, the assumption was made that temperature did not influence the spore's ability to persist. After the samples were heated in the 40°C water bath, there not only was a lack of loss, but an increase in the spore counts (8.08±0.038 CFU/mL) compared to the estimated original dose of spores, suggesting an ability of the candidate probiotic to germinate and grow at the body temperature of chickens. Then, when moved to the 75°C water bath, again there was no significant change in spore recovery (8.17±0.027 CFU/mL)

indicating that the spores were able to endure the heat in a vegetative state or sporulate efficiently enough to not be influenced by the new higher temperature.

Experiment 3.4: Effect of B. velezensis on other members of the microbiota

Table 4 shows the results of the interactions between the *B. velezensis* probiotic and a variety of potentially pathogenic bacteria or potential probiotics that can be common residents of the poultry microbiota. Of the 18 strains of bacteria tested against *B. velezensis*, three were significantly inhibited in the presence of the probiotic according to the Chi square analysis. All three significantly affected bacteria were strains of *Enterococcus cecorum*, an opportunistic pathogen in poultry that is the causative agent of enterococcal spondylitis in adult broilers. Enterococcal spondylitis causes formation of abscesses at the free thoracic vertebrate, impairing bird health and inducing lameness. Further exploration into this inhibition could show that the *B. velezensis* probiotic has more broad applications than initially thought as a preventative, with the capacity to control outbreaks of necrotic enteritis and enterococcal spondylitis.

Experiment 3.5: Gastrointestinal Survival Assay

The results of the simulation digestive assay (**Table 5**) show that as the initial concentration of spores (5.2±0.048 CFU/g) travels through the gastrointestinal tract, there is a slight decrease in concentration at the crop portion (5.17±0.029 CFU/g), that is carried through to the proventricular portion (5.05±0.044 CFU/g) and culminates overall in a statistically significant (p-value<0.01) decrease in the concentration of spores reaching the small intestines (4.85±0.039 CFU/g). While the difference in starting concentration of spores to the concentration of those that reach the small intestines is statistically significant, when the values are looked at

closer there is only a 0.35-fold change in the recovered Log_{10} CFUs, which when evaluated from a systemic and biological perspective will not likely translate to a significant decrease in the probiotics ability to colonize the small intestines overall.

Experiment 3.6: Gastrointestinal Efficacy Assay

The final step of the *in vitro* portion of this study was to ensure that the anti-clostridial properties of the *B. velezensis* probiotic were maintained as it traveled through the gastrointestinal tract. Based on the results obtained, presented in **Table 6**, the probiotic was not able maintain a statistically significant (p-value=0.112) decrease in the amount of *C. perfringens* recovered at the conclusion of the assay when compared to the non-treated group. While the difference was not statistically significant, it is approaching a level of significance (p=0.112) and when the average Log_{10} CFU/g recovery of *C. perfringens* are compared between the two groups a nearly one-Log_{10} CFU/g change is observed which correlates to a nearly 90% reduction in the clostridial load that would be present in the gut. Thus, while perhaps not an ideal candidate for treatment of disease, the probiotic has potential as a prophylactic measure to reduce *C. perfringens* in the gastrointestinal tract and prevent necrotic enteritis because it is generally an opportunistic pathogen that causes disease when it overgrows in the small intestine. A 90% reduction may be enough to prevent such activity.

Conclusions

The *B. velezensis* probiotic exhibits very potent, broad spectrum anti-clostridial effects as exhibited by the tests presented here. Additionally, the anti-clostridial effects of the probiotic can inhibit strains of *C. perfringens* known to exhibit antimicrobial resistance genes, providing

function where antibiotics were falling short. The display of anti-clostridial effects ensured that the novel *B. velezensis* strain was a candidate for use as a probiotic. Since the novel strain was able to germinate and sporulate to colonize the gastrointestinal tract and survive the pelleting process at a feed mill it would be able to survive when used as a feed additive. Based on the lack of negative interactions with beneficial bacteria, and the ability of the *B. velezensis* strain to reach the desired site of colonization while maintaining its anti-clostridial properties makes it a candidate for use in the control and prevention of necrotic enteritis in broiler flocks. The novel *B. velezensis* strain exhibits all the necessary attributes, *in vitro*, for it to be considered a candidate for use in birds against outbreaks of necrotic enteritis. The results of these experiments suggest that *in vivo* tests may show beneficial effects in preventing necrotic enteritis in broilers.

Table 1. Zone of inhibition in agar overlay inhibition assay. *B. velezensis* was initially grown on LB agar, followed by a soft agar overlay of *C. perfringens* strains. After overnight anaerobic incubation, zones of growth inhibition were measured from the edge of *Bacillus* colony to the edge of clostridial growth in mm. Values with different superscripts indicate significant differences ($p<0.05$).

Strain	Mean zone of inhibition (mm)
Control	0 ± 0^{f}
641	8.33 ± 0.13^{d}
ATCC 10543	10.08 ± 0.21^{c}
ATCC 13124	9.72 ± 0.18^{c}
BB	13.36 ± 0.36^{b}
CP1	10.23 ± 0.22^{c}
CP2	15.87 ± 0.44^{a}
Todd	10.74 ± 0.21^{c}
TXAM 011610	7.08 ± 0.12^{e}
TXAM 020410	7.13 ± 0.14^{e}
SEM	1.3351
p-value	<0.0001

Table 2. Zone of inhibition in preventative inhibition test. Bacterial treatments were initially grown on LB agar, followed by a soft agar overlay of *C. perfringens* TXAM 020410. Filter paper was submerged into antibiotic treatments and placed on an LB agar plate to dry, followed by a soft agar overlay of *C. perfringens* TXAM 020410 After overnight anaerobic incubation, zones of growth inhibition were measured from the edge of *Bacillus* colony to the edge of clostridial growth in mm. Values with different superscripts indicate significant differences ($p<0.05$).

Zones of inhibition against *C. perfringens* TXAM 020410	
Treatment	Mean size (mm)
Control	0 ± 0^{e}
B. velezensis	8.36 ± 0.2^{b}
B11	2.25 ± 0.072^{d}
B. subtilis	6.31 ± 0.083^{c}
Avilamycin	0 ± 0^{e}
BMD	0 ± 0^{e}
Metronidazole	6.97 ± 0.34^{c}
Penicillin	14.37 ± 0.58^{a}
E. coli huff	1.46 ± 0.11^{d}
SEM	1.6528
p-value	<0.0001

Table 3. Log_{10} CFU recovery after germination and sporulation. Spores were suspended in diluent along with sterile feed then heated at 40°C. A subsample was removed then the replicates were heated in a separate water bath at 75°C. Another subsample was removed. Subsamples were plated for probiotic recovery and no statistical difference was found ($p<0.05$)

Germination and Sporulation	
Temperature	Mean Log_{10} CFU/mL
40°C	8.08±0.038
75°C	8.17±0.027
SEM	0.045
Prob > |t|	0.0957

Table 4. Interaction of *B. velezensis* with other microbiota. The *B. velezensis* probiotic was streaked down the center of an LB agar plate and allowed to dry. Three of the other strains of microbiota being tested against it were then streaked across the probiotic and allowed to dry. Plates were incubated overnight according to growth needs, and signs of inhibition were noted. Significance is indicated by bolded text.

Probiotic inhibition of common intestinal bacteria			
Bacteria	Chi^2	Bacteria	Chi^2
E. cecorum 09Txs DRR	Not Significant	*S. enterica* Kentucky	Not Significant
E. cecorum 11Txs DRR	Not Significant	*S. enterica* Heidelberg	Not Significant
E. cecorum 11Txb DRR	Not Significant	*S. enterica* Typhimurium	Not Significant
E. cecorum 09Txb KK	Not Significant	*S. enterica* Enteritidis	Not Significant
E. cecorum 11Txb KK	**Significant**	*B. subtilis* TRAP/MPP/HMGB1	Not Significant
E. cecorum 09Txs KK	**Significant**	B11-*Pediococcus*	Not Significant
E. cecorum 11Txs KK	**Significant**	B11-*Lactobacillus*	Not Significant
E. coli LG	Not Significant	p-value	<0.05
E. coli Huff	Not Significant		
E. coli 122E2	Not Significant		
E. coli #2LB	Not Significant		

Table 5. Log_{10} CFU recovery across simulation digestive assay. 10^6 spores were suspended in a weak acid and incubated to mimic the crop. The pH was reduced, and enzymes were added followed by a second incubation to simulate proventricular digestion. The pH was then raised, and more enzymes were added prior to the final incubation, mimicking the conditions of the small intestines. Spore recovery was recorded at each step based on serial dilution plating. Values with different superscripts indicate significant differences ($p<0.05$).

Log CFU/mL	
Location	Mean Log_{10} CFU
Start	5.2 ± 0.048^{a}
Crop	5.17 ± 0.029^{a}
Proventriculus	5.05 ± 0.044^{b}
Intestine	4.85 ± 0.039^{c}
SEM	0.0794
p-value	<0.0001

Table 6. Log_{10} CFU recovery of *C. perfringens* in simulation digestive assay. 10^6 *Bacillus* spores were suspended in a weak acid and incubated to mimic the crop. The pH was reduced, and enzymes were added followed by a second incubation to simulate proventricular digestion. The pH was then raised, and more enzymes were added along with thioglycolate and 10^8 CFU of *C. perfringens* TXAM 020410 prior to the final incubation, mimicking the conditions of the small intestines. *C. perfringens* recovery was recorded after the final incubation based on serial dilution plating.

Clostridial Digestive Assay	
Treatment	Mean Log_{10} CFU
Control	7.93±0.47
Probiotic	7.03±0.26
SEM	0.45
p-value	0.1122

Chapter 4.

Persistence of *Bacillus* spores in the gastrointestinal tract of broiler chicks

Abstract

One of the primary factors that goes into determining if a bacteria would function well as a probiotic, is viability. To ensure the resilient nature displayed *in vitro* was maintained in live birds, a study was developed to ensure the persistence of the spores while in the GIT of a chicken. Two treatments were established, the gavage treatment received a single dose of 10^6 spores on day of hatch and the feed treatment received a consistent supply 10^6 spores per gram of feed. Beginning 24 hours after the initial dose, and every subsequent 24 hours for 5 days, 10 birds per treatment were euthanized and had their crop, ileum, and ceca aseptically collected. 12 pooled fecal samples per treatment per day were also collected to observe changes in spore passage. At all time points (24, 48, 72, 96, and 120 hours after initial dosing) and across all sampled locations the group consistently fed the spores had a significantly higher (p-value<0.01) spore recovery than the gavaged group and more consistent recovery across all locations in the gut. This indicated the probiotics ability to complete the life cycle of germination and sporulation, showing they are active in the GIT of broilers from day of hatch and must be fed on a consistent basis to maintain their presence.

Materials and Methods

Animal Housing and Handling

The *in vivo* persistence experiments took place under approved animal care protocols from the Ohio State University Institutional Animal Care and Use Committee. Ross 708 male broiler chicks were obtained on day of hatch. Chicks in the experiment were kept in two separate rooms based on treatment, in two wire floor pens per treatment with twenty-five birds per pen. Feed and water were provided *ad libitum* per the nutritional requirements established by the Nutrient Requirements of Poultry: Ninth Revised Edition, and ambient temperature was maintained within an age-appropriate range for the five-day duration of the experiment. Birds were kept under 24h of light. Chicks received the same composition of basal diet, except for the inclusion of the B. *velezensis* probiotic in one diet and not the other. The treatment that did not receive the probiotic in the diet received it on day of hatch as a single dose gavage.

Bacterial Treatment Preparation

The dried stock of probiotic spores used in this experiment was determined to be at an initial concentration of 10^{11} spores per gram of product. For the gavage treatment, 0.0012g of spores were suspended in 30mL of sterile saline to get a concentration of $4x10^6$ spores per mL of sterile saline. Each bird received an oral gavage dose of 0.25mL of the solution, for a dose of 10^6 spores per chick. For the feed treatment, 5 kg of feed was first sterilized and then had 0.05 g of spores added to it before being thoroughly mixed. The resulting concentrations was 10^6 spores per g of sterile feed.

Overview of experimental Design

In the experiment, two treatment groups housed in separate rooms were established with 2 pens per treatment and 25 day of hatch chicks housed in each pen. The first treatment received a single gavage dose of 10^6 CFU of spores on day of hatch before being placed in their pens. The second treatment received the probiotic consistently in their feed for the duration of the studies at a concentration of 10^6 CFU of spores per gram of feed. All feed was sterilized prior to being given to the birds, with the probiotic being added to the feed treatment following sterilization. Starting 24 hours after the initial dosing with the probiotic, and every subsequent 24 hours for the first 120 hours after exposure, 5 birds per pen were euthanized and had their crop, ileum, and ceca aseptically collected for CFU/g enumeration to determine spore recovery within each section of the gut. Additionally, 12 pooled fecal samples were collected from each treatment daily to determine spore passage. All collected samples were diluted in a sterile 0.9% saline + 0.1% Tween + 10mmol EDTA solution, heated in a water bath at 75°C for 15 minutes to remove any vegetative cells, and then serially diluted and plated on LB agar for CFU/g enumeration. The experiment was performed in two replicates, with the only difference being the switching of the rooms the birds were housed in to reduce the impact of room effects on the conclusions being drawn from the collected data.

Statistical Analysis

Analysis of the changes in Log_{10} CFU concentration of bacteria were analyzed using a one-way ANOVA in JMP Pro 14 statistics using the Tukey-Kramer method for the comparison of means with a statistical significance set to $p<0.05$.

Results and Discussion

Replicate 1 (**Table 7**) showed that regardless of where in the gastrointestinal tract samples were collected, or what length of time passed, the group that received the probiotic in their feed had a statistically higher concentration of spores able to be recovered, indicating a good probability that the probiotic was able to complete an entire life cycle of germination and sporulation. The only place this is not true is for the ileum, ceca, and fecal data collected 96 hours after initial exposure as these samples in the feed treatment became contaminated, preventing a comparison from being made. Replicate 2 (**Table 8**) showed the same results as seen in the first iteration of the experiment, without any of the samples becoming contaminated, showing that the concentration of spores was not dependent on the rooms the birds were housed in, but the treatment they were assigned. When comparing the colonization of the crop across both experiments between the two treatments there is an observable difference in the volume recoverable spores and the persistence of recoverable spores, with both feed treatments being significantly higher in concentration and more consistent in the probiotic's persistence. These observable differences stand true for the small intestines and the ceca, with the feed treatment consistently outperforming the gavage treatment in both volume of spores present and persistence of those spores over time. The ceca of the gavage treatment had higher recovery of the probiotic than the crop and small intestines in the gavage group but was still significantly lower when compared to the feed treatment. When the fecal samples between the two treatments across both experiments were compared (**Figure 1**), the feed treatment again showed a higher volume of spores passed in the feces more consistently over time than the gavage group was able to, showing the differences in persistence that was occurring within the gastrointestinal tract of the chicks in the different treatments.

Conclusion

Since the probiotic was able to be recovered from the gastrointestinal tract of chicks in every location sampled when fed on a consistent basis, the *B. velezensis* probiotic maintained the attributes characterized *in vitro* that showed it would be capable of passing through the digestive tract without being significantly hindered or reduced by the extreme environment. This displays that, when fed consistently, the novel *B. velezensis* strain is able to function as a probiotic, reaching the desired site of activity and persisting as a part of the gut microbiota. Moving forward, the final step will be to ensure that the anti-clostridial properties exhibited by the probiotic *in vitro* are maintained when the probiotic is used in an *in vivo* necrotic enteritis challenge model.

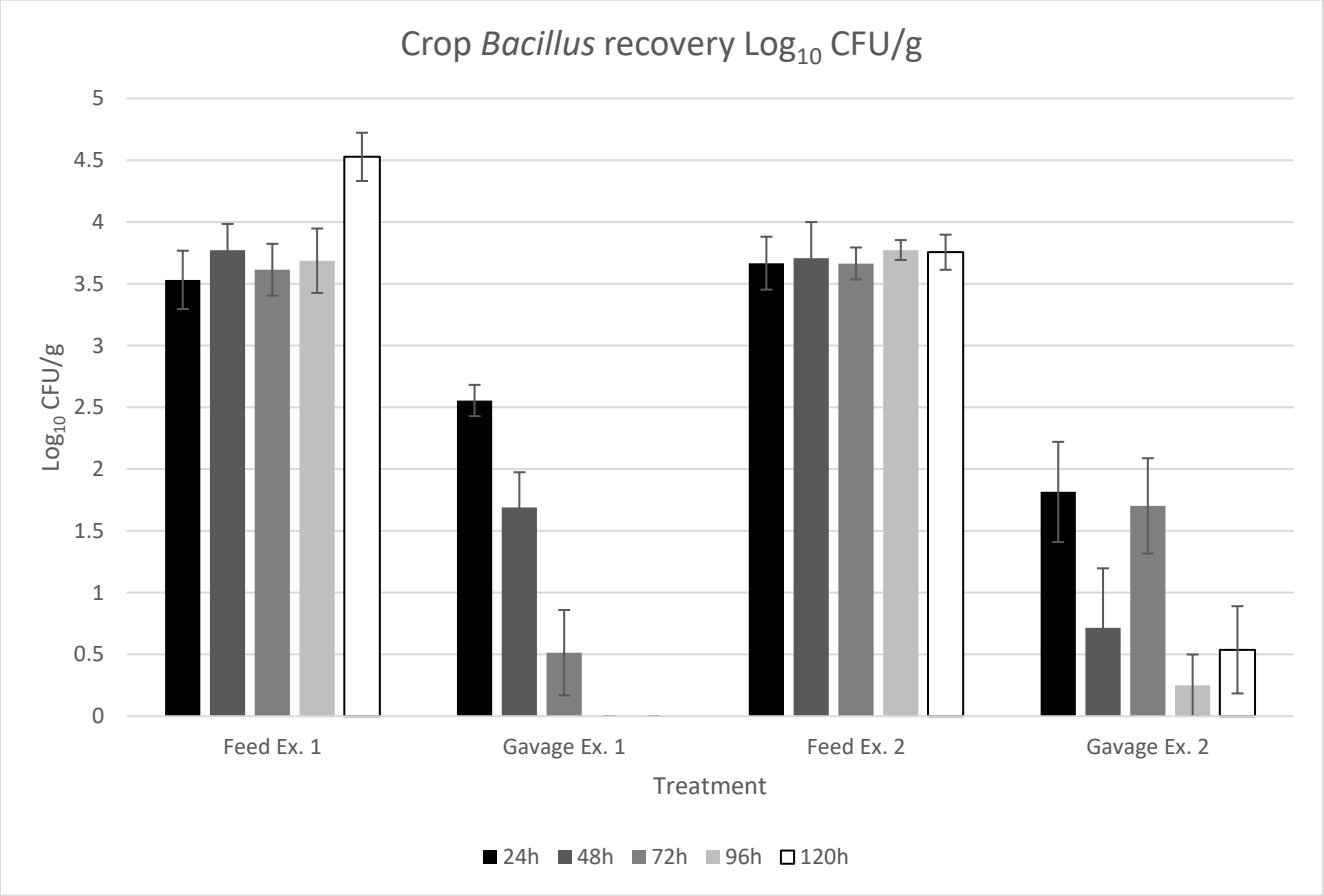
Table 7. Persistence of spores in the GIT of broilers that either consumed *Bacillus* in feed or by oral gavage on day of hatch, Experiment 1. Differences in the recovery of spores between the feed treatment and gavage treatment across all three sampled portions of the gastrointestinal tract at all five-time points sampling was performed. A suspected error in sample processing affected 96h time collection. Values represent mean ± standard error.

Time	Location	Feed Log_{10} CFU/g	Gavage Log_{10} CFU/g	p-value
24h	Crop	3.53±0.27	2.56±0.27	0.0027
	Ileum	3.8±0.37	2.18±0.37	0.0003
	Ceca	3.95±0.27	2.93±0.27	0.0015
48h	Crop	3.77±0.36	1.69±0.36	<.0001
	Ileum	4.72±0.32	2.22±0.32	<.0001
	Ceca	4.66±0.26	2.47±0.26	<.0001
72h	Crop	3.61±0.4	0.52±0.4	<.0001
	Ileum	4.64±0.36	1.03±0.36	<.0001
	Ceca	4.06±0.37	1.65±0.37	<.0001
96h	Crop	3.69±0.26	0±0.26	<.0001
	Ileum	0±0.27	0.4±0.27	0.1679
	Ceca	0±0.39	1.16±0.39	0.0159
120h	Crop	4.53±0.2	0±0.2	<.0001
	Ileum	4.84±0.11	0±0.11	<.0001
	Ceca	5.01±0.42	0.93±0.42	<.0001

Table 8. Persistence of spores in the GIT of broilers that either consumed *Bacillus* in feed or by oral gavage on day of hatch, Experiment 2. Differences in the recovery of spores between the feed treatment and gavage treatment across all three sampled portions of the gastrointestinal tract at all five-time points sampling was performed. Values represent mean ± standard error.

Time	Location	Feed Log_{10} CFU/g	Gavage Log_{10} CFU/g	p-value
24h	Crop	3.67±0.46	1.82±0.46	0.0021
	Ileum	4.42±0.5	2.01±0.5	0.001
	Ceca	4.57±0.37	3.25±0.37	0.0032
48h	Crop	3.71±0.56	0.72±0.56	0.0002
	Ileum	4.61±0.49	1.51±0.49	0.0002
	Ceca	4.43±0.44	2.59±0.44	0.0027
72h	Crop	3.66±0.41	1.7±0.41	0.0011
	Ileum	4.59±0.57	1.75±0.57	0.0009
	Ceca	4.43±0.34	3.45±0.34	0.0159
96h	Crop	3.77±0.26	0.25±0.26	<.0001
	Ileum	4.31±0.48	0.86±0.48	<.0001
	Ceca	4.53±0.37	2.19±0.37	0.0002
120h	Crop	3.76±0.38	0.54±0.38	<.0001
	Ileum	4.26±0.52	1.61±0.52	0.0002
	Ceca	4.76±0.38	2.18±0.38	<.0001

Figure 1. Comparison of crop spore recovery between feed and gavage, Exp. 4.1 and 4.2. *Bacillus* spores were provided continuously in feed at 10^6 CFU/g or by oral gavage of 10^6 CFU/bird on day of hatch. Crops from 5 chicks per group were aseptically collected daily for quantification of *Bacillus* spores.



Chapter 5.

Discussion and Conclusions

The novel *B. velezensis* strain was of interest due to the exhibition of anti-clostridial activity in a preliminary screening test upon discovery and isolation. Further investigation was needed to characterize the extent and efficacy of the anti-clostridial properties displayed, survivability of the strain when applied as a probiotic, practicality of its use when considered as a small part of the greater overall microbiota, and viability of this strain to persist through the gastrointestinal tract while maintaining anti-clostridial properties exhibited within the *in vitro* experiments presented here. Throughout Chapter 3, the novel probiotic was run through a variety of rigorous experiments to characterize its anti-clostridial properties and potential ability to survive in the GIT. The first experiment confirmed and expanded upon the perceived exhibition of anti-clostridial properties, *in vitro*, of the novel *B. velezensis* strain. In testing the strain against nine different, known pathogenic, strains of *C. perfringens* the broad-spectrum effects were documented and allowed the strain to move on to the next experiment.

The next step aimed at comparing the antimicrobial effects of the novel probiotic against some commonly used treatments and preventatives to gauge its efficacy. In establishing both antibiotic and bacterial treatments the experiment was able to display the efficacy of the novel probiotic and allowed a comparison to be drawn against existing treatments as well as exhibited the strains' ability to circumvent the presence of antimicrobial resistance genes that other treatments were stifled by. While this experiment gave a relatively good display of the efficacy of the probiotic, some aspects must be kept in mind while drawing conclusions. The first being that since the probiotic treatment was allowed to grow overnight on an agar plate prior to the administration of the clostridial challenge, there is no way of determining the concentration of

antimicrobials produced by it so a true comparison cannot be drawn against the antibiotics which were administered at defined and controlled concentrations. Another aspect to consider is that there is no way of determining if the agar was able to stifle or exacerbate the ability of any of the antimicrobial's abilities to diffuse, thus influencing the size of the zone of inhibition. Finally, it is necessary to keep in mind that when evaluating the results for BMD and avilamycin, these antibiotics are meant to be administered in feed which could influence their ability to function *in vitro* as their solubility could be affected.

The germination and sporulation assay aimed to show that the *B. velezensis* strain could survive both the internal body temperature of a chicken and the high heat process of pelleting experienced at the Ohio State feed mill. While the data shows a lack of change in recovery, accompanied by a small increase in spore counts when going from the 40°C water bath to the 75°C water bath, there is no way to say with certainty whether the probiotic was able to complete a full cycle of germination into a vegetative cell at 40°C and then sporulation back into spore form upon entering the 75°C water bath. Additionally, the feed mill at Ohio State runs its pelleter at a lower temperature than is normal in the industry due to the lower quantity of feed they tend to handle which should be investigated further to ensure the spore can persist in the higher pelleting temperature.

When evaluating the effects of the novel *B. velezensis* strain on other common members of the microbiota, a heavy focus was placed on bacteria that commonly act as opportunistic pathogens in poultry along with some strains that have the potential to act as probiotics. While this information is helpful in characterizing the probiotics role as a preventative, it is far from comprehensive. To observe the full impact that the *B. velezensis* strain has on the microbiota, next generation sequencing would need to be performed, with the focus placed on changes in the

families *Clostridiaceae, Enterobacteriaceae, Enterococcaceae Lactobacilliaceae,* and *Baciliaceae* among others.

The gastrointestinal survival and efficacy assays were developed to form an idea of how the probiotic would function in the digestive tract of a bird. Each simulated portion of the experiment was formulated to mimic the corresponding section of the digestive tract as closely as could be done, *in vitro.* The initial survival assay was used to determine whether the probiotic would be killed of before reaching its desired site of activity. While there was a statistically significant reduction in the number of spores reaching the small intestines, the reduction would not be expected to produce a biologically relevant decrease in activity. Additionally, the observed decrease could have been a result of the increase in volume that accompanied each step of the assay when altering the pH of each solution. When looking at the maintenance of the anti-clostridial properties as the probiotic traveled through the gastrointestinal tract, a decrease in *C. perfringens* that was approaching statistical significance was observed. While the probiotic failed to produce a statistically significant decrease in *C. perfringens* in the assay, the fact remains that a nearly one-fold Log_{10} was observed which would be expected to produce a biologically relevant outcome when account is given to the nearly 90% reduction in *C. perfringens* present in each replicate.

Chapter 4 details the experiment performed to confirm the ability to survive the gastrointestinal tract characterized *in vitro*, ensure the persistence of the spores throughout the gastrointestinal tract and passage in feces, and compare two means of delivery, a single dose gavage treatment and a constant fed treatment in feed. In both replicates of the experiment, the ability of the spores to survive the gastrointestinal tract was displayed by both treatments, confirming the results obtained *in vitro*. When comparisons were drawn between the two

treatments, it was apparent, across all time points and every sampled location of the gastrointestinal tract as well as feces, that the spores were able to pass through the gastrointestinal tract without much reduction, but their colonization of the gastrointestinal tract was relatively low, which is to be expected of a *Bacillus* species. As a result, the best way to ensure the probiotic persists in the gastrointestinal tract is to provide it in feed on a consistent basis, allowing it to always be present and exhibit beneficial effects consistently on the bird.

Throughout the duration of this study a wide variety of methods were used, manipulated, and developed to characterize the novel strain and its potential for use as a probiotic. Moving forward, the final step in characterizing the potential of this novel *B. velezensis* strain is to perform a full-scale necrotic enteritis challenge model *in vivo*. In doing this, the ability of the probiotic to not only prevent the incidence of necrotic enteritis, but also the effects that it will have on overall bird performance can be observed and allow characterization of the true probiotic potential this novel strain carries.

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