VALIDATION OF THE FUNG DOUBLE TUBE TO ENUMERATE *CLOSTRIDIUM PERFRINGENS* FROM THE INTESTINAL CONTENTS OF BROILER CHICKENS RAISED ON DIFFERENT DIETS

by

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Abstract

Clostridium perfringens causes necrotic enteritis (NE), resulting in decreased feed efficiency and increased mortality, costing the poultry industry USD 2 billion a year worldwide. The objective of the first trial was to validate the Fung Double Tube (FDT) to detect and enumerate *C. perfringens* in chicken intestines. Two methods (FDT and petri plates) and three media (Shahidi Ferguson Perfringens [SFP] with egg supplement, polymyxin B [p], and kanamycin [k; E]; SFP with p and k [P]; and SFP with cycloserine [C]) were arranged in a 2 x 3 factorial, resulting in six treatments. The FDT with medium C (5.35 log CFU/g) had significantly ($P<0.05$) higher *C. perfringens* counts than any other media/method combination. The objective of the second and third trials was to determine the effect of diet type on the population of *C. perfringens* in broiler intestines using the FDT. Trial 2 tested: corn-soybean meal (SBM), low-crude protein (19.8%)/high synthetic amino acids (SAA), and barley (56%)-fishmeal (4%; BF). Diets in Trial 3 included: corn-SBM, barley (7.46%), fishmeal (4%), and BF. Diets in Trial 2 contained an antibiotic and a coccidiostat; diets in Trial 3 did not. After 21 days, birds in Trial 2 fed BF had significantly higher ($P<0.05$) counts (5.96 log CFU/g) of *C. perfringens*, as compared to all other diets. Both, corn-SBM and SAA diets resulted in 3.89 log CFU/g. In Trial 3, birds fed the corn-SBM diet (2.7 log CFU/g) had significantly lower ($P<0.05$) counts than broilers fed BF (4.15 log CFU/g). When broilers were fed fishmeal (3.583 log CFU/g) and barley (3.577 log CFU/g) separately, *C. perfringens* counts were numerically higher compared to the corn-SBM diet, but numerically lower than birds fed BF. Barley and fishmeal inclusion increased the incidence of *C. perfringens*, and their combination resulted in a cumulative effect. The FDT method is able to detect *C. perfringens* at higher levels than the conventional petri plate method ($P<0.001$) and it also proved to be an effective method to detect differences in *C. perfringens* counts from the intestines of chickens fed different diet.

Table of Contents

List of Figures

List of Tables

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Dedication

I would like to dedicate this work to my parents, Alecia Godoy and Diego R. Barrios, as well as my brother, Diego A. Barrios. Los quiero mucho

Chapter 1 - Literature Review

Introduction

The Fung Double Tube (FDT) is a method created by Fung in 1980. It was developed due to the increasing need for a more convenient way to anaerobically incubate samples. The method consists of two glass tubes of different diameters; one smaller diameter tube is inserted into a larger diameter glass tube containing molten agar. This method was used only with anaerobic agar to obtain anaerobic viable cell counts (Fung and Lee, 1980).

The FDT has been used as a selective system for the proliferation of *Clostridium perfringens*. The FDT has been adapted to be used with food samples to detect and enumerate *C. perfringens*. Ali *et al*. (1991) compared four rapid methods (Oxyrase enzyme, FDT, GasPak Anaerobic System, and the anaerobic petri dish) to isolate three different strains of *C. perfringens* from inoculated meats. For every strain, the FDT method resulted in a greater colony count as compared to the three other plate methods.

Clostridium perfringens is a Gram-positive bacterium. This microorganism is ubiquitous; it is commonly found in soil, water, decaying organic matter, gastrointestinal tracts of multiple species, and feces (Craven *et al.*, 1998; Tschirdewahn *et al*., 1991). Since it is spore forming, *C. perfringens* is very resilient to high temperatures and conditions as well as toxic chemicals (Paredes-Sabja, D. *et al.*, 2008; Craven *et al*., 2001). It is the causal microorganism for Necrotic Enteritis (NE) in poultry (Fukata *et al*. 1998). *Clostridium perfringens* types A and C, along with some predisposing factors, have been found to cause lesions in the small intestine and ceca of chickens (Immerseel *et al.*, 2004). *Clostridium perfringens* is of great interest to the poultry industry due to the potential detrimental growth effects it may have in a flock, even at subclinical levels (Rehman *et al.,* 2006). *Clostridium perfringens* is also one of the most common causes of foodborne illness in humans (Heikinheimo *et al.*, 2004). The Centers for Disease Control and Prevention (CDC, 2012) estimates that nearly one million people are affected every year, making *C. perfringens* the third most frequent source of domestically acquired foodborne illness after Norovirus and *Salmonella*.

There are multiple elements that affect the proliferation of *C. perfringens* in chicken intestines, one of the most important factors being diet formulation (Choct, 2009; McDevitt *et*

al., 2006). Some feed ingredients have been found to exacerbate the numbers of *C. perfringens* in the gastrointestinal tract of chickens. Diets formulated with wheat resulted in increased NE intestinal lesion scores when compared to broiler chickens fed a corn-based diet (Branton *et al*., 1997). In another study, Drew *et al.* (2004) investigated the effects of protein source on the intestinal populations of *C. perfringens* in broiler chickens. Diets were formulated to contain 230, 315, and 400 g/kg of fishmeal or soy protein concentrate (SPC). Numbers of *C. perfringens* in the ileum and ceca increased when the amount of protein increased from 230 to 400 g/kg.

The purpose of this research study was to validate the Fung Double Tube for use with chicken intestinal contents. Currently, the industry uses the standard petri plate method to detect and enumerate *C. perfringens*. Authors that have studied the FDT have established that it is simple, rapid, inexpensive, and more selective than petri plates. This thesis is divided in three chapters. The first chapter contains a brief literature review. The second chapter attempts to elucidate the advantages of the FDT over petri plates using three different agar media. In the third chapter, different diets were formulated and fed to broiler chickens over the course of three weeks to determine the effects in numbers of *C. perfringens* in the small intestine.

Clostridium perfringens

Clostridium perfringens is a Gram-positive, spore forming, anaerobic, rod-shaped bacterium (Rehman *et al*., 2006). This encapsulated, non-motile microorganism is fastidious in growth requirements (Wise and Siragusa, 2005). Most often, complex media like cooked meat or thioglycollate broth are used as enrichment (Labbe, 1991). It is regularly found in the gastrointestinal tracts of healthy humans and animals. Welch and Nuttall first identified *C. perfringens* in 1892 as *Bacillus aerogenes capsulatus* (Hatheway, 1990). In Great Britain it was commonly known as *C. welchii* and sometimes-called Frankel's bacillus in Germany, until it was designated *C. perfringens* by Bergey (Freeman, 1979). There are five types of *C. perfringens*: A, B, C, D, and E. Each can be identified by toxin production, and a distinction should be made between toxins and the enterotoxin *C. perfringens* type A produces, which causes food poisoning in humans (Varnman *et al.*, 1996). The four most significant extracellular toxins are: alpha, beta, epsilon, and iota. *Clostridium perfringens* has been described to produce eight other toxins, three (delta, theta, kappa) of which can be lethal, but these are seldom involved in disease origin (McDonel, 1986). All strains produce alpha toxin. *Clostridium perfringens* type B and C produce

beta toxin. Type B and D produce the epsilon toxin. Type E produces the iota toxin (Ali *et al.*, 1991). *Clostridium perfringens* type A and C are the only ones associated with foodborne disease in man as well as necrotic enteritis in fowl. Type B causes lamb dysentery, and sheep/goat/guinea pig enterotoxemia. Type D causes enterotoxemia in sheep, and pulpy kidney disease in lambs. Type E causes enterotoxemia in calves, lamb dysentery, and guinea pig/rabbit enterotoxemia (Hatheway, 1990).

Several investigators have determined growth temperature ranges for *C. perfringens*. Labbe *et al.* (1991) established that *C. perfringens* can reproduce at temperatures between 15-50°C. This range is similar to Hatheway (1990), in which they reported between 20-50°C. Varnman *et al.* (1996) verified these temperatures stating that growth is severely restricted at temperatures below 15 to 20°C. Hence, proper refrigeration temperatures (below 10°C) can be an effective means of control. *Clostridium perfringens* is mesophilic; it grows best at temperatures that are neither too hot nor too cold (25°C - 60°C). The optimum range is between 37-47°C and at these temperatures, mean generation time, the time required for the bacterial count to double, is approximately 10-12 minutes (Murray *et al*., 2009). Generation time depends on a variety of factors including: strain, pH, temperature, water activity, and redox potential (Labbe and Juneja, 2006). One *C. perfringens* strain (NCTC 8238) showed the shortest generation time reported for bacteria, 7.1 minutes at 41°C. An eight-strain composite of *C. perfringens* was tested for generation times in different meat products and one growth medium (raw beef strips, ground beef, and fluid thioglycollate). When incubated at 45°C, generation times ranged from 8.5 min in ground beef to 12.2 min in thioglycollate (Ali *et al.*, 1991). These short generation times allow the bacteria to outcompete other microorganisms that may need similar resources in a certain environment.

Clostridium perfringens optimum pH range is between 5.5-7.0 (Hickey and Johnson, 1981). However, it can grow at a pH as low as 5 and as high as 9. At these extreme pHs, other factors such as temperature and the nature of the acid-producer become more important to allow growth (Labbe, 1991). It is essential to note that these pHs are similar to those found in many food products, especially meat products. In live broiler chickens, the pH in the small intestine has been determined to be between 6.00-7.78. When two different diets were fed, a standard corn-soybean meal (SBM) diet and one supplemented with molasses, it was concluded that there were

no differences in pH with either diet. Furthermore, it was apparent that the GI tract could overcome any diet to maintain a stable pH (Wiseman *et al*., 1956).

Clostridium perfringens is commonly found in the GI tract of broiler chickens, humans, and other mammals (Pedersen *et al*., 2003). When intestinal samples of broiler chickens were analyzed for *C. perfringens*, 75-95% tested positive (Immerseel *et al*., 2004). Drew *et al.* (2004) determined that *C. perfringens* is normally found at ~10^4 colony-forming units (CFU)/g of broiler digesta. These results agree with Jia *et al*. (2009) who stated that *C. perfringens* is present at low levels in healthy poultry. Necrotic enteritis becomes a problem when *C. perfringens* counts reach 10^7-10^8 CFU/g (Cooper and Songer, 2009). Lindstrom *et al.* (2011) collected data from different human feces studies concerning the prevalence of *C. perfringens*. Multiple authors found prevalence to be between 57-94% in research done in different parts of the world. Lastly, *C. perfringens* is routinely found in the environment. It is found in soil, water, and other organic materials. As far as poultry facilities, *C. perfringens* has been isolated from litter, dust, walls, floors, fans, transportation coops, feeders, and feed (Craven *et al*., 2001a; Craven *et al*., 2001b).

Necrotic Enteritis

Necrotic enteritis in poultry is caused by *C. perfringens* types A and C (Williams *et al*., 2003). Parish (1961) first described the disease in cockerels in England. Some of the symptoms include depression, reluctance to movement, ruffled feathers, somnolence, diarrhea, loss of appetite, and anorexia (Helmboldt and Bryant, 1971). Mortality ranges from 0-50% (Cooper *et al*., 2009) have been reported in infected flocks. Since then, virtually every area that raises poultry has reported signs of necrotic enteritis. Long *et al.* (1974) proposed the pathogenesis for NE. First, epithelial cells are vacuolated, and the epithelium lifts off the lamina propria, which is congested and edematous. These lesions can be caused by a combination of factors like, toxin production and/or coccidiosis. *Clostridium perfringens* cells attach to the lamina propria where it thrives and the tissue becomes necrotic as large numbers of heterophils, a type of phagocyte, flood the foci (sites of lesions). A combination of disease inducing factors such as, bacteria proliferation, heterophil lysis, and necrosis of the villus seem to develop quickly. The inflammation zone then becomes riddled with mononuclear cells, cells containing lymphocytes and antigen-presenting cells, and eosinophilic-staining (proteinaceous) amorphous material. This necrotizing process moves from the tip of the villi to the crypt. In chronic cases, villi may be

found to have multiple cysts form recurrent necrosis. In birds that overcome the disease, injured epithelial cells are replaced by newly formed reticular structures. These new cells travel from the crypt to the tip of the villi and replace the old, damaged cells. The result is a short, flat villus with reduced surface area for nutrient absorption (Parish, 1961; Long *et al*., 1976). These morphologically altered villi are the necrotic lesions found in the field and some *C. perfringens* challenge trials (Figure 2). The acute form of NE results in enlarged lesions along the gut wall, and the epithelium becomes eroded and detached; consequently, a diphteric membrane is formed. This yellow, green, or brownish pseudo-membrane is called the "Turkish towel," which describes the appearance of the friable, gas-filled, foul-smelling GI tract (Williams, 2005).

When investigators attempted to create a model to reproduce NE in a laboratory setting, they realized that inoculation of *C. perfringens* alone did not cause the disease found in the field (Pedersen *et al*., 2007). Therefore, it was assessed that certain cofactors must play a significant role in the pathogenicity of *C. perfringens*. Williams (2005) reviewed concurrent infections of coccidiosis and necrotic enteritis in chickens (Figure 1). The copious interactions of these diseases with predisposing factors, control methods, sources of infection, and disease form is a testament to the complexity of this poultry industry matter. This review will focus on two main predisposing factors: coccidiosis and diet formulation. Shane *et al.* (1985) noted that several authors had considered coccidiosis to be a predisposing factor for NE and proceeded to describe the pathogenesis of *Eimeria acervulina*, one of the protozoa responsible for coccidiosis in poultry. When the oocysts are ingested, they quickly attach to the intestinal wall causing lesions where the protozoa reproduce numerous times. These are the lesions to which *C. perfringens* attaches.

Poultry producers are not only concerned with the acute form of NE. Recent studies have shown that the subclinical form of the disease can be as detrimental as the acute illness (Heier *et al*., 2001). Lovland *et al.* (1999) stated that this symptomless disease is often overlooked at the farm, and the effects are only noticed at the processing facility. Subclinical NE (SNE) can cause cholangiohepatitis, a condition where the liver is enlarged with pale reticular patterns and sometimes small, pale foci. In the United Kingdom, it was estimated that 4% of broiler carcasses and 12% of livers are condemned at processing plants as a consequence of clostridial infection; thereby, reducing profit (McDevitt *et al*., 2006). Moreover, sparse lesions that may be found in a

case of SNE may be enough to hinder growth performance; thus resulting in an underproductive flock (Mitsch *et al*., 2004).

Feed Ingredients

It has been reported that diet formulation has the greatest impact on the prevalence of *C. perfringens* in chicken GI tracts (Yegani and Korver, 2008). The poultry industry formulates diets on a least-cost basis, which may become problematic if nutritionists do not take into consideration the pathological consequences that some ingredients may have in the GI tracts of chickens. Every feed ingredient has a specific purpose in the diet. For instance, cereal grains are fed for their energy concentration as well as fiber. Also, some grain and animal/plant meals are used for their protein content. Since these ingredients are obtained from different sources, they are highly variable in macro and micronutrients (Bedford, 1996).

Authors have studied the effects of grain inclusion on gut microbiota, and it is well established that small cereal grains such as, barley, rye, and wheat tend to increase the prevalence of *C. perfringens* in the GI tract. Shakouri *et al.* (2008) investigated the influence of barley, sorghum, wheat, and corn on counts of *C. perfringens* in the different intestinal segments. Corn and wheat had the lowest *C. perfringens* counts, followed by sorghum, while barley yielded the highest counts. These findings agree with Riddell and Kong (1992).

Researchers have concluded that the increase in gut viscosity and increased chyme transit time elicit the overgrowth of *C. perfringens* in the intestines (Klasing, 1998). Grains like wheat and barley contain high amounts of non-starch polysaccharides (NSP), which increase viscosity (Jia *et al*., 2009). Furthermore, it has been alleged that since these grains are high in NSP, the bird cannot absorb nutrients as efficiently; thereby, leaving them for microbes like *C. perfringens* to consume (Langhout *et al.*, 1999).

Shakori *et al*. (2008) and Jia *et al.* (2009) also studied the impact of several diets with the inclusion of a blend of carbohydrases such as, glucanase and xylanase. Their findings suggested that enzyme addition did not affect counts of *C. perfringens* in the different intestinal sections. However, they did find an improvement in growth performance. They stated that enzymes improved chyme viscosity by degrading the encapsulation of nutrients in diets.

For this reason, researchers have investigated the use of enzymes in wheat and barley based diets on the incidence of *C. perfringens* in chicken intestines. Jackson *et al.* (2003) studied

the effect of beta-mannanase addition on flocks infected with *Eimeria* spp. and *C. perfringens*. They found that feeding this enzyme significantly reduced the impact of *C. perfringens* on the performance of infected flocks as well as intestinal lesion scores. Moreover, the authors explained that this might be due to beta-mannanase crossing the intestinal wall to provoke an immune response. Hofacre *et al.* (2003) found similar results when birds were fed manna-oligosaccharides. They determined that this enzyme tended to ameliorate the symptoms of necrotic enteritis, but not significantly. A marked effect was only found when mannan-oligosaccharides were included along with lactic acid producing, competitive exclusion products (probiotics).

Feed form has also been investigated on the incidence of *C. perfringens*. When birds were fed whole wheat as compared to ground, researchers found reduced counts of *C. perfringens* in the gut (Bjerrum *et al.*, 2005). These results can be extrapolated to the findings of Engberg *et al.* (2002). They found that when birds were fed coarse versus fine mash or pellets, *C. perfringens* counts were always higher in flocks fed mash diets. These authors concluded that feeding pellets or whole grains increases gizzard activity, which consequently triggers hydrochloric acid production and decreases pH in the GI tract. This drop in pH of approximately 0.5 units may be responsible for decreased *C. perfringens* counts.

Another well-established fact is that the *C. perfringens* population can be affected by animal protein source and inclusion rates. Palliyeguru *et al.* (2011) studied the inclusion of protein concentrates (potato, fish, and soy) on subclinical NE. They concluded that the potato containing diet resulted in the highest incidence of *C. perfringens* in the gut, followed by fish, and soy. Also, the potato containing diet had the highest activity of trypsin inhibitors and lowest lipid content. Increased trypsin inhibition does not allow for the inactivation of alpha and beta toxins produced by *C. perfringens* resulting in increased intestinal wall lesions. Drew *et al.* (2004) formulated diets containing fishmeal or a soy protein concentrate at different levels. Feeding dietary fishmeal resulted in a higher incidence of *C. perfringens* as compared to the soy protein diet. Furthermore, as levels increased for both soy and fishmeal diets, counts of *C. perfringens* increased as well. A notable difference in fishmeal protein concentrate compared to the soy protein concentrate was the amino acid ratio in this experiment; the methionine and glycine ratios were 1.3 times greater in fishmeal diets. Muhammed *et al.* (1975) determined that methionine was required for *C. perfringens* sporulation. This may be of interest to nutritionists

since some authors have estimated that 10-20% of synthetic amino acids are not absorbed and reach the lower intestinal tract, i.e. ceca; thereby, aiding in proliferation of *C. perfringens*.

The effects of fat source on *C. perfringens* population remain largely unknown. Knarreborg *et al*. (2002) studied the bacterial microflora in chicken intestines after feeding different dietary fats (soy oil, and a tallow and lard mix) in rations containing antibiotic growth promoters (AGP). When soy oil was fed, *C. perfringens* counts were significantly lower than diets containing animal fats. The authors stated that since plant oils contain higher amounts of unsaturated fatty acids, the chyme in birds fed oil diets would have decreased viscosity, decreasing transit time. Furthermore, an additive effect was found when soy oil was fed along with AGP, which may be due to facilitated antibiotic dispersion caused by the lipophilic properties of the oil. Dänicke *et al*. (1999) investigated the effects of fat source on *C. perfringens.* They found that total anaerobic counts increased with animal fat addition. However, zinc bacitracin was included in their diets, which specifically targets Gram-positive microorganisms like *C. perfringens*; thus, potentially biasing their results.

Antibiotics and coccidiostats have been commonly included in poultry diets since the mid-1940s and 1950s (Yegani and Korver, 2008; Williams *et al*., 2003). Prescott *et al*. (1978) studied the inclusion of zinc bacitracin to prevent necrotic enteritis and concluded that it successfully controlled the *C. perfringens* challenge. Flocks in the antibiotic treatments were able to overcome disease and perform similarly to unchallenged birds. These results have been replicated by multiple authors using different antibiotics such as, virginiamycin and salinomycin (George *et al.*, 1982; Miles *et al.*, 1984; Bolder *et al.*, 1999; Engberg *et al.*, 2000). Improvements in flock performance with the inclusion of antibiotics and coccidiostats are well established and ubiquitous in the literature. However, the potential loss of subtherapeutic antibiotic usage in livestock in the United States of America demands research of viable alternatives to these compounds.

Lastly, multiple options have been studied to control *C. perfringens* in poultry. Some researchers have studied the inclusion of complex carbohydrates and fibers like, pine shavings, guar gum, and pectin with limited success (Branton *et al.*, 1997; Langhout *et al.*, 1999). Another popular alternative is the use of competitive exclusion-based products such as, prebiotics and probiotics (Kaldhusdal *et al.*, 2001; Geier, 2010). Still, these products failed to yield consistent results. Other options that have been investigated are the addition of lactose and organic acids

(Takeda *et al.*, 1995; Mikkelsen, 2009). Potassium diformate did not produce lowered counts of *C. perfringens*. Lactose reduced *C. perfringens* counts, but resulted in undesirable ceca characteristics including, enlargement and increased fermentation (Takeda *et al*., 1995). Therefore, many authors agree that a multifactorial approach is necessary if antibiotics are to be completely replaced by these strategies (McDevitt *et al*., 2006).

Media

A number of agar and broth media have been used to detect and enumerate *C. perfringens* since it was first described (Hauschild and Hilsheimer, 1973). The Nagler (Nagler *et al*., 1939) method consisted of the reaction between human serum and *C. welchii* (Type A). When human serum was inoculated with *C. welchii* and incubated anaerobically for 16h, the medium became turbid. Furthermore, when the broth was centrifuged, three well-demarked zones could be seen: a pellet at the bottom containing bacilli, a middle layer with opalescent serum, and a top layer of fat-like material. The authors established that the lethal toxin produced by *C. welchii* type A caused the reaction. This methodology was later modified by Nagler *et al*. (1945) to be able to use plating agar with digest media, as opposed to broth, in order to be able to produce single colonies of *C. welchii*.

Hobbs *et al.* (1953) studied food poisoning by *C. welchii* and used blood agar to detect the microorganism. They failed to specify what type of blood and how much was used to make the plating media. Currently, most laboratories use 5% defibrinated sheep or horse blood with anaerobic agar for research and diagnostic purposes. There are two complications with this medium: it is not selective, and *C. perfringens* may react differently depending on the strain and incubation time. If a mixed sample (one presumably containing multiple microorganisms) is directly plated on blood agar, all those microbes are able to grow and colony counts are not accurate due to competition for resources. Also, some strains of *C. welchii* did not show hemolysis, yet others developed hemolysis over time (Horse *et al*., 1963).

Lyons and Owen (1941) made minor modifications to the Wilson-Blair medium, in order to test several strains of *Clostridium* such as, *C. perfringens*, *C. multifermentans*, *C. tertium*, *C. oedematiens*, C. *novyi*, *C. sporogenes*, *C. tetani*, among others. *Clostridium perfringens* produced isolated zones of blackening due to the ability of *C. perfringens* to reduce sulfite to sulfide, resulting in the deposit of a black ferrous sulfide. They pointed out that this medium was

successful at allowing growth of *C. perfringens* as well as other strains of *Clostridium*, but could not differentiate between toxigenic and non-toxigenic strains.

McClung and Toabe (1946) researched the addition of an egg yolk supplement to their medium formulation, which included proteose peptone, sodium phosphate, glucose, and agar, instead of Nagler's previously described modification. This media resulted in a stronger reaction due to the alpha toxin produced by *C. perfringens*. The colonies were round, smooth and surrounded by a white, opaque zone. They also noted that the Nagler reaction was not specific (Hayward, 1941, 1943; Crook, 1942). Other microorganisms like *C. parabotulinum*, *C. sorelli, C. bifermentans, C. hemolyticum*, and *C. novyi* produced a similar reaction to that seen with *C. perfringens*. McClung and Toabe (1946) concluded that the use of this method could presumptively identify *C. perfringens*. Angelotti *et al.* (1962) proposed another method for detection and enumeration of *C. perfringens*. They further adapted an already modified Wilson and Blair medium (Thompson, 1939) by adding sulfadiazine, which had been reported to suppress the growth of other black zone-producing colonies such as, *Enterobacteriaceae*. The inclusion of sulfadiazine in the sulfite-polymyxin-sulfadiazine (SPS) medium, allowed the quantification of *C. perfringens* from plates, as opposed to previous media that could only be used to indicate presumptive *C. perfringens* colonies due to the unspecific nature of the agar. They also proposed a motility-nitrate medium for confirmation of black colonies. Shahidi *et al.* (1971) developed the Shahidi Ferguson Perfringens (SFP) agar. They proposed a lactose-motility (LM) agar for confirmation of black colonies presumed to be *C. perfringens*. *Clostridium perfringens* is non-motile and ferments lactose, unlike other *Clostridium* species; therefore, *C. perfringens* is confirmed when lactose changes color and there is no evidence of motility. These authors had previously determined that the SPS agar used by Angelotti *et al.* (1962) sometimes failed to produce black colonies, and even inhibited some strains of *C. perfringens*. Shahidi *et al.* (1971) tested multiple strains of *C. perfringens* on SFP and SPS agars and the recovery rates were 90.6% and 69.8%, respectively. When food samples were processed for *C. perfringens* using SFP and SPS agars and confirmed using LM agar, 27 samples showed positive results with SFP as compared to 5 samples with SPS. Harmon *et al*. (1971) tested SFP and Tryptose-Sulfite-Cycloserine (TSC) for *C. perfringens* recovery. TSC was prepared with the same basal ingredients as SFP, but D-cycloserine was added as the antibiotic, whereas SFP uses polymyxin and kanamycin. The authors concluded that even though SFP had 95% recovery rate for *C.*

perfringens, TSC was more selective (90% recovery). Increased selectivity is a desirable characteristic in testing media since other microorganisms sometimes interfere with the detection of *C. perfringens* from samples containing background microflora. Hauschild and Hilsheimer (1973) made one more modification to the TSC medium used by Harmon, which tested the viability of TSC without the addition of the egg yolk emulsion it required. They argued that this ingredient further complicated media preparation and also hindered selectivity. When the authors tested TSC with and without egg yolk emulsion, the egg yolk emulsion-free agar resulted in a better recovery rate.

Since TSC accuracy still allows room for improvement, researchers have proposed confirmation methods for *C. perfringens* such as, LM agar (Shahidi *et al.*, 1971). For this work, the novel RapID-ANA II (Innovative Diagnostic Systems, Inc., Atlanta, Ga.) system was chosen. The RapID-ANA II is a miniaturized test, which relies on 18 enzyme reactions to accurately identify certain anaerobic microorganisms including *C. perfringens* (Marler *et al*., 1991). Celig and Schreckenberger (1991) evaluated the RapID-ANA II system as compared to conventional methods to identify different anaerobes. The RapID-ANA II system identified *Clostridium* spp. with 94% accuracy. Moreover, 100% of *C. perfringens* strains were correctly confirmed.

Currently, private companies as well as the U.S. Food and Drug Administration use SFP and TSC interchangeably. Additionally, the use of egg yolk emulsion varies depending on the researcher's own laboratory experience with different strains of *C. perfringens* and food samples.

Anaerobic Methodologies

Numerous anaerobic systems have been designed and proposed to detect anaerobic microorganisms, such as *C. perfringens*. Most of the methods used rely on samples plated on petri plates and then incubation in some type of anaerobic chamber. For instance, plates are often incubated in glove boxes, anaerobic jars, and gas pack systems to achieve the oxygen-free environment required by anaerobic microbes. This review will focus on the AnaeroPack (Mitsubishi Gas Chemical America, Inc., New York, N.Y.) system. Van Horn *et al.* (1997) evaluated this system against other jar and pouch methods. The AnaeroPack system relies on a sachet placed directly in a jar. Upon exposure to air, the sachet swiftly scavenges oxygen to less than 0.1% within an hour, producing an atmosphere containing 20% CO_2. The AnaeroPack sachets used with the AnaeroPack jar performed as well or better than any other plate system

tested. It is also much easier to use because it does not require a catalyst to achieve an anaerobic environment and it does not depend on a screw-cap system that has been shown to fail more often than the rectangular jars used by the AnaeroPack (Van Horn *et al.*, 1997).

Still, methods requiring these chambers are very expensive and necessitate a substantial initial investment. Glove boxes are costly to buy and maintain, and are also cumbersome to use. Anaerobic jars and gas packs require the purchase of the chambers themselves and the substrates used to scavenge oxygen have to be acquired every time the chamber is used.

For this reason, novel methods have been developed that exclude anaerobic chambers and samples can be placed directly in a regular incubator. Some of these techniques include the Miller-Prickett tube (Miller *et al*., 1939), the pouch method (Bladel *et al.,* 1964), the Fung Double Tube (Fung and Lee, 1980), Oxyrase enzyme addition (Ali *et al*., 1991), and the agar bottle plate (Hermann *et al*., 1986). All these methods aim to mitigate the initial and subsequent investment of chamber-dependent systems. Also, these innovative solutions are intended to be simpler and more anaerobe selective.

In 1980, Fung created the Fung Double Tube method in Lee's laboratory during a research project in Taiwan. Fung and Lee (1980) describe the method as follows. The FDT consists of a sterile, glass test tube (15cm x 1.5cm OD; Kimax 45066) containing 10 mL of tempered agar. A sample, most often one milliliter, is pipetted into this tube. Then, a smaller diameter (15cm x 1.0cm OD; Kimax 450421), sterile, glass test tube is inserted into the "outer" tube creating a thin, translucent layer. The "outer" tube is then capped and an anaerobic environment is immediately achieved. The agar scavenges any oxygen when there is headspace left in the FDT. When Fung and Lee (1980) introduced this methodology, they used anaerobic agar to determine total anaerobic counts. They obtained higher counts with the FDT as compared to the petri plate method, although log counts were similar. The FDT produced greater anaerobiosis, it was easier to prepare and clean, it was more compact and light, and it eliminated the use of hot paraffin required by petri plates.

Ali *et al.* (1991) compared four different anaerobic systems: FDT, Oxyrase enzyme, GasPak Anaerobic System, and anaerobic petri dish (APD). Multiple strains of *C. perfringens* in nutrient broth and ground beef were inoculated into all four systems and enumerated after incubation at 37^{o}C. With all *C. perfringens* strains, the FDT produced the highest counts in the shortest amount of time. Black colonies were visible after only six hours and maximum growth

was reached between 8-10 hours. The FDT was also the least expensive of all systems. These findings were confirmed by Sabah and Fung (2002). They used the FDT with TSC with and without egg yolk emulsion as well as SFP with egg yolk emulsion and determined that there were no differences in recovery rates among all three media. Fung *et al.* (2007) validated the use of the FDT with water samples to determine fecal contamination in recreational waters of Hawaii. Ruengwilysup *et al.* (2008) investigated the use of PCR as the confirmation technique for the FDT. They argued that routinely used biochemicals to confirm presumptive *C. perfringens* colonies might sometimes yield flawed results. This may be due to: bacteria producing small amounts of enzyme, decreased enzyme strength, and/or insufficient amount of cells in the colonies tested. Polymerase Chain Reaction (PCR) is superior because it does not depend on any of these factors. However, in their research, 97 out of 147 isolates were identified as *C. perfringens* with biochemicals, as compared to 99 with the PCR test. The last modifications made to the FDT method were by Vijayavel *et al.* (2009). Samples were treated with high-temperature and short time (HTST – 70^{o}C, 2.5 min), and 4-methylumbelliferyl-phosphate (MUP) was added to SFP and TSC. Pretreatment with HTST decreases vegetative background-interfering microflora and elicits germination of *C. perfringens* spores to shorten growth time in the FDT. Fluorescent MUP had previously been used as a confirmation method for *C. perfringens* in TSC plates. Colonies of *C. perfringens* show blue fluorescence under UV light (320-400nm) in the presence of MUP. Inclusion of MUP into TSC in FDT further saved identification time by fusing the diagnostic and confirmation procedures, and also made the FDT more selective for *C. perfringens*.

Research Objectives

This research study had two main objectives:

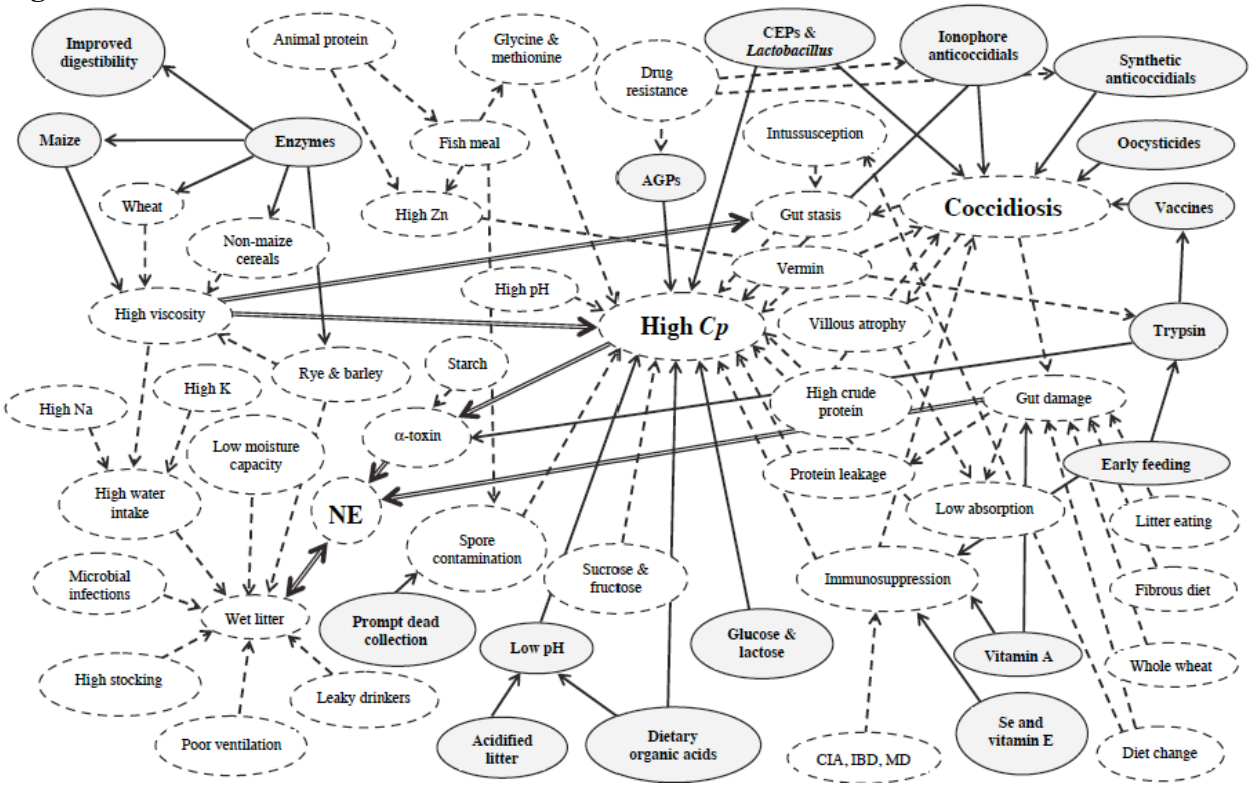
1- To validate the use of the Fung Double Tube (FDT) to detect and enumerate *C. perfringens* from samples of broiler chicken intestines.

2- To apply the FDT to enumerate *C. perfringens* in broilers fed a set of experimental diets presumed to promote the proliferation of *C. perfringens* in chicken intestines.

The first study consisted of three agar media tested in petri plates and FDT, resulting in six different treatments. The same intestinal sample was plated in all six treatments and colonies presumed to be *C. perfringens* were enumerated.

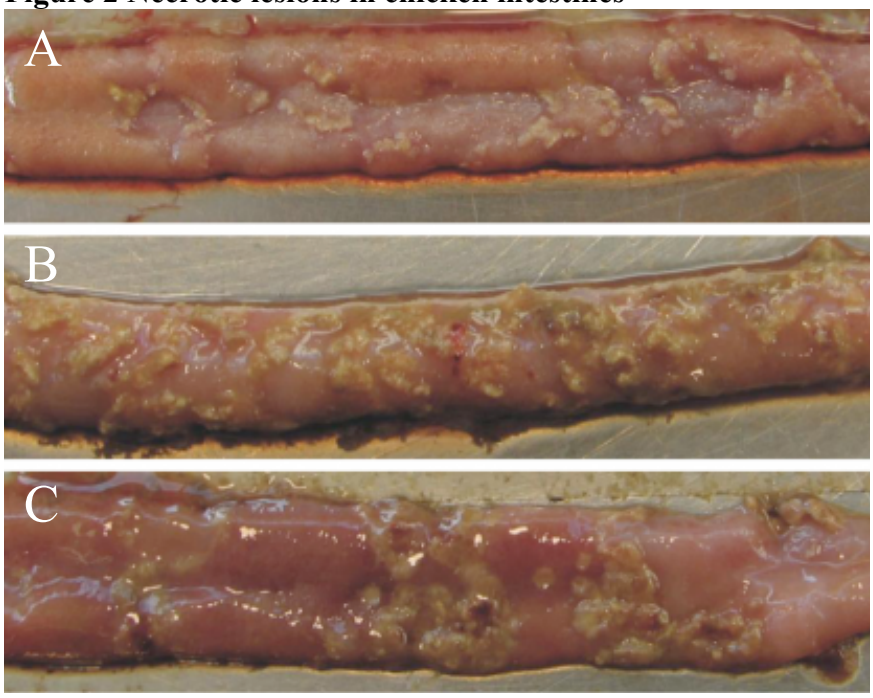
The second set of studies was performed to determine the effect of feed ingredients on the numbers of *C. perfringens* in broiler chicken intestines. Two trials were conducted. For each trial, there were four diets (treatments) and three pens per treatment. At the end of each trial, birds were selected and *C. perfringens* in intestinal samples was enumerated using the FDT.

Figure 1 Interaction between coccidiosis and NE with environmental factors



Solid-line arrows are beneficial in controlling disease. Dashed-line arrows impart high disease risk factors. Double-line arrows depict major disease-risk factors. AGP, antibiotic growth promoter; CIA, chick infectious anemia; CEP, competitive exclusion product; Cp, *Clostridium perfringens*; IBD, infectious bursal disease; MD, Marek's disease; NE, necrotic enteritis. (Williams, R.B. 2005).

Figure 2 Necrotic lesions in chicken intestines



Yellowish necrotic lesions in three intestinal samples. Intestines A and C show a few marked lesions. Intestine B shows clusters of lesions typical of the "Turkish towel" syndrome. (Source: http://www.mdpi.com/2072-6651/2/7/1913/htm. Accessed: April 26, 2012).

Chapter 2 - Comparison of Three Agar Media in Fung Double Tubes and Petri Plates to Detect and Enumerate *Clostridium perfringens* in Broiler Chicken Intestines

Abstract

Clostridium perfringens is an anaerobic, spore-forming bacterium, which causes necrotic enteritis (NE), resulting in decreased feed efficiency and increased mortality in chickens. It is estimated that *C. perfringens* infects nearly one million people in the US every year. The purpose of this study was to validate the Fung Double Tube (FDT) to detect and enumerate *C. perfringens* in chicken intestines. Nine broilers were selected and euthanized at days 21 and 42 for a total of 18 samples. The jejunum and ileum from every broiler were harvested and inoculated into two methods and three different media, resulting in a 2 x 3 factorial for a total of six treatments. The two methods were FDT and petri plates, and the three media consisted of: Shahidi Ferguson Perfringens (SFP) with egg supplement, polymyxin B (p), and kanamycin (k; E); SFP with p and k (P); and SFP with cycloserine (C). At 21 days, counts using medium C with FDT (4.51 log CFU/g) and plates (2.38 log CFU/g) were higher ($P<0.05$) than using media E or P. On day 42, there were no differences among plate treatments and medium E had the highest counts (0.98 log CFU/g). Out of all the FDT, medium C (5.35 log CFU/g) had the highest counts, followed by medium P (3.54 log CFU/g). This study illustrates that the FDT method is able to detect *C. perfringens* at higher levels ($P<0.001$) than the conventional petri plate method; therefore, the FDT can be implemented and further explored by laboratories around the world.

Key words: *Clostridium perfringens*, Fung Double Tube, broiler, anaerobic method

Introduction

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming bacterium, which is often found in water, dust, floors, fans, and walls of poultry houses (Craven *et al.,* 2001a,b). Along with certain predisposing factors like, coccidiosis and small grains, *C. perfringens* is the causative agent of Necrotic Enteritis (NE). This disease results in decreased feed efficiency and increased mortality in poultry flocks (Shakouri *et al*., 2008). The poultry industry is most concerned with the subclinical form of necrotic enteritis (SNE), which often goes undiagnosed by poultry producers resulting in diminished profit potential (Heier *et al*., 2001). Necrotic enteritis costs the poultry industry approximately $2.6 billion every year worldwide (McDevitt *et al*., 2006). *Clostridium perfringens* is one of the most common causes of foodborne illness in the United States of America, affecting nearly one million people every year (CDC, 2012). Therefore, rapid and accurate detection and enumeration of this microorganism is of the utmost importance.

Multiple methods have been developed to detect and enumerate *C. perfringens* from different types of samples such as, water, foods, and animal specimens. *Clostridium perfringens* produces beta hemolysis when plated on 5% sheep blood agar. It is because of this characteristic that multiple research and diagnostic laboratories have historically used blood petri plates to identify *C. perfringens*. Unfortunately, some strains of *C. perfringens* have been shown to lack the ability to produce hemolysis (Hauschild *et al*., 1973). Shahidi Ferguson Perfringens (SFP) and Tryptose Sulfite Cycloserine (TSC) are media that rely on the principle that *C. perfringens* reduces sulfites to sulfides, producing black colonies that are easily recognized. Hauschild *et al*. (1973) investigated SFP, SFP without egg yolk supplement, and TSC and concluded that TSC resulted in the highest counts of *C. perfringens* and the highest selectivity. Also, the addition of egg yolk supplement made enumeration of *C. perfringens* colonies more cumbersome.

Clostridium perfringens is generally recognized as a fastidious microorganism. Thus, methods that are more efficient in identifying *C. perfringens* should be thoroughly investigated. Fung and Lee (1980) found that the FDT resulted in higher total anaerobic counts than anaerobic petri plates. Ali *et al*. (1991) successfully used TSC in FDT to study the incidence of *C. perfringens* in food samples such as, ground beef and ground turkey. Vijayavel *et al.* (2009) studied the use of the FDT with SFP and SFP with a phosphatase test with environmental water samples. The authors determined that the FDT was the only system available capable of

producing counts of *C. perfringens* after only 5-6 hours of aerobic incubation, proving the FDT system to be a valid, rapid system for *C. perfringens* detection and enumeration.

The use of the FDT to grow *C. perfringens* from chicken intestinal samples has not been studied. Therefore, the purpose of this experiment was to test three different media (SFP, SFP without egg yolk supplement, and SFP with cycloserine) and two methods (FDT and conventional petri plates) to determine which is more effective in detecting and enumerating *C. perfringens.*

Materials and Methods

General Procedures

Animal Care

The Kansas State University Institution of Animal Care and Use Committee approved all experimental procedures. This trial was conducted in the late summer (2011) using day-old Cobb 500 (Cobb-Vantress, Siloam Springs, AR) male chicks. Chicks were housed in floor pens (1.52 x 1.66 m) on clean, pine wood shavings at the Thomas B. Avery Poultry Research Unit (Manhattan, KS). There were 60 chicks per pen, and three replicates, totaling 180 birds. A ziggity nipple water drinking system was set up in each pen and was manually adjusted as birds grew to ensure the watering system was kept at a proper level. One metal hanging feeder was placed in each pen. The chicks were kept under 24 h of light for the first three days and 23L:1D for the duration of the experiment. Broiler chicks were maintained at 33^{o}C for the first three days and temperature was raised by 2.5^{o}C every seven days until the culmination of the study. Diets were formulated to meet or exceed nutrient concentrations recommended by the NRC (1994; Table 2.1). Chicks were grown from d 0 to 42 with feed and water provided *ad libitum*. Deceased birds were removed as necessary. On d 21 and 42, nine birds were randomly selected and euthanized by cervical dislocation for a total of 18 samples.

Sampling

Broilers were necropsied and the jejunum (bile duct entrance to Meckel's diverticulum) and ileum (Meckel's diverticulum to ileo-cecal junction) were quickly harvested. The jejunum and ileum were cut longitudinally, and along with any chyme, placed in a filtered stomacher bag, weighed, and diluted (dilution factor = 2) with 0.1% peptone water (Difco) and stomached (Seward 400, Worthing, Great Britain) for two minutes. Then, ten-fold serial dilutions were made with 0.1% peptone water (Difco). Every sample was then inoculated into both methods (FDT and petri plates) with all three media described below.

Agar Media

The agar media used in FDT and petri plates were: SFP (Difco) with antimicrobic vial k (kanamycin; Difco), antimicrobic vial p (polymyxin B; Difco), and egg yolk enrichment 50% (Difco; E); SFP with kanamycin (Difco) and polymyxin B (Difco; P); and SFP (Difco) with D-cycloserine (Sigma-Aldrich) to make TSC agar (C). All agars were tempered to 45^{o}C before antimicrobial and egg yolk enrichment addition. Antimicrobials for SFP (Difco) preparation were added per Difco instructions. TSC was prepared by adding 0.4 mg D-cycloserine (Sigma-Aldrich)/L of SFP (Difco).

Fung Double Tube and Petri Plate Preparation

The FDT were prepared by sterilizing the capped outer tubes (15cm x 1.5cm OD; Kimax 45066) separately from the inner tubes (15cm x 1.0cm OD; Kimax 450421; Fig 2.1). The inner tubes were placed in a rack, wrapped in aluminum foil, and sterilized. After autoclaving the media, 25 mL of molten (45^{o}C) agar were pipetted into the outer tube. FDT were kept in water baths at 45^{o}C until inoculation. When samples were ready to be plated, one-mL was inoculated into the FDT. The FDT were inverted three times to distribute the sample in the medium. The inner tube was then aseptically inserted using forceps, thus creating a thin agar layer. Then, the outer tube was capped. The FDT were placed directly in the incubator at 37^{o}C for 24 hours. After incubation, large (~2mm) black colonies were enumerated and recorded in order to obtain colony-forming units (CFU) per gram of intestinal sample (Figure 2.2).

Petri plates were prepared the day before sampling. A 0.1 mL sample was plated and spread using 'L' shaped spreaders (Fisher Scientific). Plates were placed in an anaerobic jar (Mitsubishi Gas Chemical America Inc., New York, NY) along with a sachet, which upon

exposure to air scavenges oxygen to produce an anaerobic atmosphere that contains approximately 20% CO_2 within one hour. After 24 hours of incubation at 37^{o}C, large (~2mm) black colonies were enumerated and recorded to calculate colony-forming units (CFU) per gram of intestinal sample.

Confirmation Procedure

After samples were appropriately enumerated and recorded, two black colonies presumptive for *C. perfringens* were picked for confirmation. First, the inner tube was removed exposing the agar layer. Then, a piece of agar containing the desired colony was carefully extracted from the outer tube using disposable, sterile loops. Each colony was streaked for isolation on CDC anaerobe 5% sheep blood agar (BD Diagnostic Systems) and anaerobically incubated as previously stated for 24 hours. Colonies exhibiting beta-hemolysis were picked and a lawn was streaked on CDC anaerobe 5% sheep blood agar (BD Diagnostic Systems). Plates were anaerobically incubated as previously stated for 24 hours. These samples were then inoculated into RapID-ANA II kits (Innovative Diagnostic Systems, Inc., Atlanta, Ga.) and results were recorded after four hours of incubation as specified by RapID-ANA II instructions.

Statistical Analysis

The experimental data were analyzed as a completely randomized design. The log values obtained from three replications based on the variables: method (FDT and petri plates), and three media (E, P, and C) were analyzed using the MIXED procedure in SAS (Release 9.1 for Windows, SAS Institute, Cary, NC). Broiler chickens were the experimental unit and an alpha of 0.05 was used to establish significance. Least square means were calculated, and interactions among media, method, and sampling day were determined.

Results

Preliminary studies were carried out to better understand the FDT method compared to the conventional petri plate method. Preliminary results indicated that colony formation could be observed in the FDT after only eight hours of incubation, although total colony count was not achieved until 24 hours of incubation. Hence, 24 hours was chosen as the optimal incubation time for broiler chicken intestinal samples inoculated into the FDT. Petri plates were difficult to examine at different time intervals since they were placed in stacks inside anaerobic jars. Also,

C. perfringens colonies on petri plates showed deficient growth and visual examination was difficult as compared to the FDT after 24 hours of incubation.

No clinical signs of necrotic enteritis were observed in the flock over the course of the study. Counts of *C. perfringens* in the jejunum and ileum were obtained at 21 and 42 days. There were no obvious signs of acute NE (Turkish towel) in the GI tract observed during sampling. Although no three-way interaction was found among day (21 vs. 42), media (P, E, C), and method (FDT vs. petri plates), data is first presented separated by day since there are numerical differences in *C. perfringens* counts on days 21 and 42.

Clostridium perfringens counts for day 21 are shown in Table 2.2. Medium C resulted in significantly higher ($P<0.05$) counts than media E or P when the FDT was used. As for media in petri plates, medium C resulted in significantly higher ($P<0.05$) counts, while medium P produced the lowest counts (0.76 log CFU/g). When comparing the FDT with the petri plate method, medium P in the FDT detected over one log more colonies (1.66 log CFU/g) than plates. Agar E in petri plates produced low counts (0.74 log CFU/g), and its FDT counterpart resulted in 1.59 log CFU/g. Counts using medium C with the FDT (4.51 log CFU/g) were significantly higher ($P<0.05$) than counts obtained with petri plates (2.38 log CFU/g).

Counts for *C. perfringens* for day 42 are displayed in Table 2.3. There were no significant differences among all media in petri plates and medium E had the highest counts (0.98 log CFU/g). Out of all the FDT, medium C had significantly higher ($P<0.05$) counts (5.35 log CFU/g) than medium P (3.54 log CFU/g) and medium E had significantly lower ($P<0.05$) counts (1.89 log CFU/g) than medium P. When medium C was used in petri plates, counts were significantly lower (0.91 log CFU/g) than its FDT (5.35 log CFU/g) counterpart ($P<0.05$). The same effect was found when medium P was used in the FDT as compared to petri plates.

As previously mentioned, no interaction was found among sampling day, media, and method in our data. Thus, it is valuable to interpret our results focusing on the method by media interaction. Counts of *C. perfringens* for both sampling days were averaged and the results are shown in Table 2.4. Medium E used in both the FDT and petri plates resulted in the lowest *C. perfringens* counts of any other media and method combination. Medium P in the FDT had significantly higher ($P<0.001$) *C. perfringens* counts than petri plates. Medium P was numerically higher than medium E in FDT. *Clostridium perfringens* counts with medium C in the FDT were significantly higher ($P<0.001$) than all other method and media combinations.

Overall, the FDT detected significantly higher ($P<0.001$) counts of *C. perfringens* compared to the conventional petri plate method.

Results obtained through the confirmation procedure with RapID-ANA II kits for day 21 are shown on Table 2.4. The rapid kits identified (No. of samples): *Clostridium innocuum* (4)*, C. clostridioforme* (3)*, C. tertium* (3)*, C. butyricum* (2)*, C. perfringens* (2)*, C. beijerinckii*, and *Clostridium* spp. Twenty-two other codes entered were identified as unacceptable. Samples from day 42 resulted in the following identifications: *Clostridium innocuum* (16)*, C. tertium, C. butyricum, C. baratii, C. novyi*, and *Lactobacillus minutus* (2). The rest of the samples (40) were identified as unacceptable.

Discussion

Several authors have successfully employed the FDT method to detect and enumerate *C. perfringens* from different types of samples such as, ground beef and ground turkey, and animal clinical specimens (Sabah and Fung, 2002; Ruengwilysup *et al*., 2008). Fung and Lee (1980) found that the FDT recovered higher counts of total anaerobic bacteria than the anaerobic petri plate system. Ali *et al*. (1991) compared three anaerobic systems (FDT, Oxyrase enzyme, and GasPak Anaerobic System) to recover *C. perfringens* from food samples and determined that the FDT recovered more *C. perfringens* than any other method. In all these studies, researchers agree that the FDT was able to recover higher counts of *C. perfringens* because of its ability to achieve an immediately anaerobic environment. These studies are consistent with our results. When broiler intestines were analyzed for *C. perfringens* using three media (E, P, and C) with the FDT and conventional anaerobically incubated petri plates, the FDT resulted in higher counts across all samples.

Researchers have studied a number of agars for the proliferation of *C. perfringens*. Hauschild and Hilsheimer (1973) studied the two most often used media in petri plates, SFP and TSC, to determine which one was more effective in detecting and enumerating *C. perfringens*. The authors concluded that SFP was less selective and cumbersome to prepare. Also, SFP was harder to enumerate due to the lack of discernable halos produced by *C. perfringens* and large spreading colonies that merged single colonies. The results obtained in our study concur with Hauschild and Hilsheimer (1973) and show that TSC obtained the highest counts of *C. perfringens* as compared to SFP and SFP without egg yolk supplement. Furthermore, SFP

without egg yolk supplement performed better than SFP. This is in contrast to Sabah and Fung (2002) who investigated the use of the FDT with SFP and TSC with meat products and peptone water and did not find significant differences between the two media.

Previous authors have investigated different methods to confirm black colonies, presumably formed by *C. perfringens* in SFP and TSC media, such as Lactose-Motility agar (Hauschild and Hilsheimer, 1973), Polymerase Chain Reaction (Ruengwilysup *et al*., 2008), and phosphatase reaction (Vijayavel *et al*., 2009). The RapID-ANA II was chosen for this experiment due to its rapidity, efficacy, novelty, and simplicity. Other researchers showed high levels of accuracy (up to 100%) when identifying certain strains of *C. perfringens* from stock cultures (Celig and Schreckenberger, 1991; Marler *et al*., 1991). Although, Ruengwilysup *et al*. (2008) argued that tests that rely on biochemical reactions like, RapID-ANA II, may be unreliable since bacteria may sometimes produce minute amounts of enzyme, or it may not produce the enzyme, or the enzyme it produces may be weak, or sometimes there are not enough bacterial cells within the colonies to produce the enzyme to breakdown biochemicals in the rapid kit. RapID-ANA II also relies on a database in order to interpret the coded results. *Clostridium perfringens* is constantly evolving at one of the fastest bacterial generation times (7-8 minutes). Therefore, if the database is not regularly updated, wild strains of *C. perfringens* may remain unidentified.

The FDT is superior to other anaerobic methodologies because of its characteristic ability to immediately achieve an anaerobic environment. This quality allows for faster, more efficient recovery of injured cells. Moreover, the use of TSC with the FDT allows for increased selectivity, simpler preparation procedures, and improved enumeration. The FDT is rapid, simple, selective, reusable, and inexpensive; therefore, its potential in other areas that involve anaerobic microbiology must be investigated.

Acknowledgments

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Table 2.1 Starter and grower diet formulations and nutrient composition (%, as-fed basis)

Ingredients	Starter	Grower
Corn	57.51	66.54
Soybean meal (48%)	32.99	25.71
Porcine meat and bone meal (47.9%)	4.00	4.00
Soy oil	3.04	1.60
Limestone	0.66	0.98
Defluorinated phosphate	0.97	0.47
Salt	0.28	0.23
DL-Methionine	0.19	0.12
Feed additives[123]	0.35	0.36
Calculated Composition		
Metabolizable energy (Kcal/kg)	3200	3200
Crude protein	23.00	20.00
Lysine	1.23	1.04
Methionine	0.54	0.44
Tryptophan	0.29	0.24
Threonine	0.85	0.74
Calcium	1.00	0.95
Available phosphorus	0.45	0.35
Sodium	0.20	0.16

[1] Supplied per kg of diet: manganese, 0.02%; zinc, 0.02%; iron, 0.01%; copper, 0.0025%; iodine, 0.0003%; selenium, 0.00003%; folic acid, 0.69 mg; choline, 386 mg; riboflavin, 6.61 mg; biotin, 0.03 mg; vitamin B6, 1.38 mg; niacin, 27.56 mg; pantothenic acid, 6.61 mg; thiamine, 2.20 mg; menadione, 0.83 mg; vitamin B12, 0.01 mg; vitamin E, 16.53 IU; vitamin D3, 2,133 ICU; vitamin A, 7,716 IU.

[2] Monensin 0.099 g/kg, Elanco Animal Health, Indianapolis, IN.

[3] Bacitracin methylene disalicylate. 0.055 g/kg, Alpharma, Bridgewater, NJ.

Table 2.2 *C. perfringens* counts (log CFU/g) in broiler intestines, day 21

	FDT	Petri plates
P[1]	1.66[y]	0.76[y]
E[2]	1.59[y]	0.74[y]
C[3]	4.51[a,x]	2.38[b,x]

[a,b] Means within rows with no common superscripts differ significantly (P<0.05).
[x,y] Means within columns with no common superscripts differ significantly (P<0.05).
[1] SFP. [2] SFP w/o egg yolk sup. [3] TSC.

Table 2.3 *C. perfringens* counts (log CFU/g) in broiler intestines, day 42

	FDT	Petri plates
P[1]	3.54[a,y]	0.77[b]
E[2]	1.89[z]	0.98
C[3]	5.35[a,x]	0.91[b]

[a,b] Means within rows with no common superscripts differ significantly (P<0.05).
[x,y,z] Means within columns with no common superscripts differ significantly (P<0.05).
[1] SFP. [2] SFP w/o egg yolk sup. [3] TSC.

Table 2.4 Average (21, 42 d) *C. perfringens* counts (log CFU/g) in broiler intestines

	FDT	Petri plates
P[1]	2.6[a,y]	0.77[b]
E[2]	1.74[y]	0.86
C[3]	4.93[a,x]	1.64[b]

[a,b] Means within rows with no common superscripts differ significantly (P<0.05).
[x,y] Means within columns with no common superscripts differ significantly (P<0.05).
[1] SFP. [2] SFP w/o egg yolk sup. [3] TSC.

Table 2.5 RapID-ANA II confirmation results, day 21

Identification	No. of samples (n=38)
C. innocuum	4
C. clostridioforme	3
C. tertium	3
C. butyricum	2
C. perfringens	2
C. beijerinckii	1
***Clostridium* spp.**	1
Unacceptable	22

Table 2.6 RapID-ANA II confirmation results, day 42

Identification	No. of samples (n=62)
C. innocuum	16
C. tertium	1
C. butyricum	1
C. baratii	1
C. novyi	1
L. minutus	2
Unacceptable	40

Figure 2.1 Fung Double Tube diagram

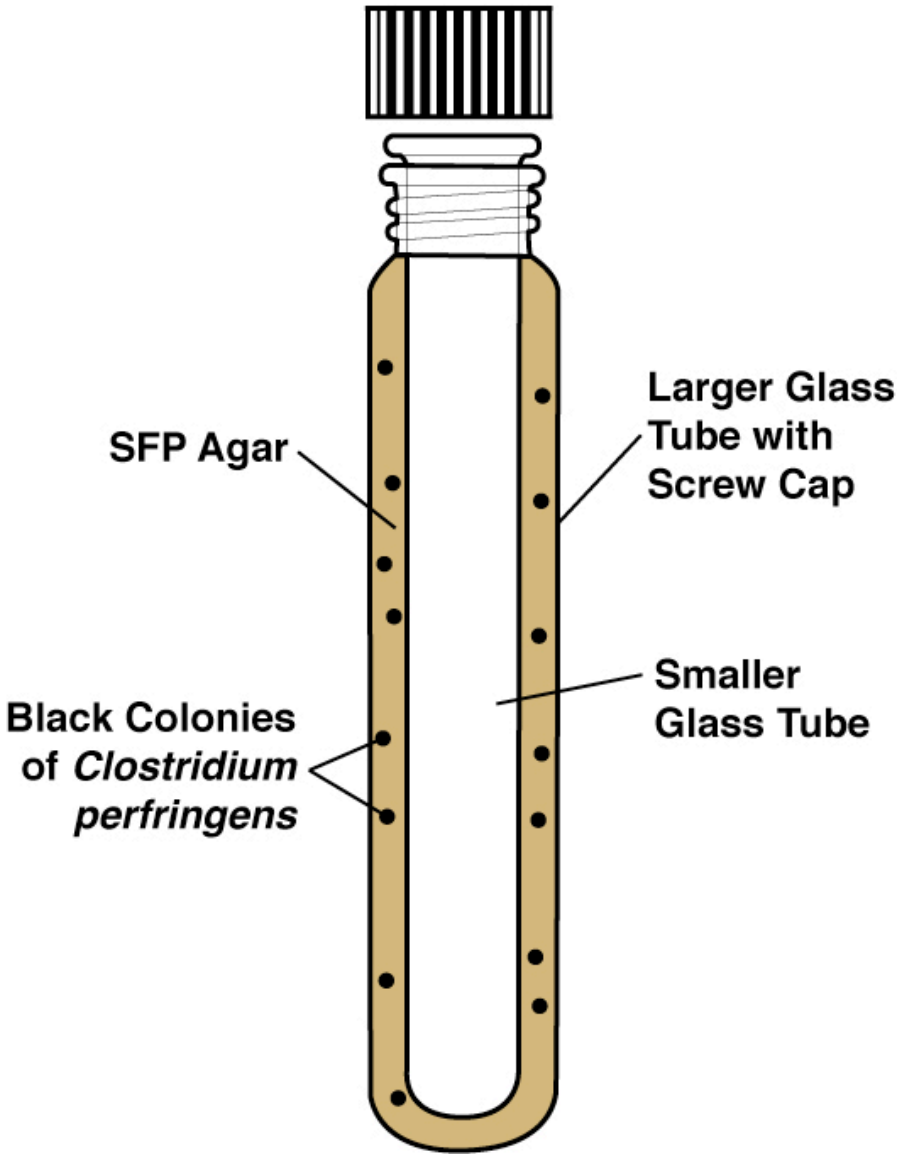

Figure 2.2 Three FDT inoculated with chicken intestinal samples after 24 h of incubation at 37^{o}C using SFP agar

Chapter 3 - The Effect of Dietary Ingredients on *Clostridium perfringens* in Broiler Chickens Intestines Using the Fung Double Tube

Abstract

Necrotic enteritis (NE) costs the poultry industry USD 2 billion a year worldwide. Subclinical NE costs as much as $0.05 per bird. *Clostridium perfringens*, the causal microorganism of NE, attaches to epithelial lesions most often caused by coccidiosis, resulting in NE. The objective of this study involving two trials was to determine the effect of diet type on the population of *C. perfringens* in broiler intestines using the Fung Double Tube (FDT). There were four diets and three replications per diet in each trial. Twenty newly hatched chicks were placed per floor pen, for a total of 240 birds per trial. Diets in Trial 1 included: corn-soybean meal (SBM), expanded corn, low-crude protein (19.8%)/high synthetic amino acids (SAA), and barley (56%)-fishmeal (4%; BF). Diets in Trial 2 consisted of: corn-SBM, barley (7.46%), fishmeal (4%), and BF. Diets in Trial 1 contained an antibiotic and a coccidiostat; diets in Trial 2 did not. After 21 days, three birds per pen were selected and the ileum and jejunum were harvested. In Trial 1, birds fed BF had significantly higher ($P<0.05$) counts (5.96 log CFU/g) of *C. perfringens*, as compared to all other diets. Both, corn-SBM and SAA diets resulted in 3.89 log CFU/g. *Clostridium perfringens* counts were the lowest (3.64 log CFU/g) in broilers offered the expanded corn ration. In Trial 2, birds fed the corn-SBM diet (2.7 log CFU/g) had significantly lower ($P<0.05$) counts than broilers fed BF (4.15 log CFU/g). When broilers were fed fishmeal (3.583 log CFU/g) and barley (3.577 log CFU/g) separately, *C. perfringens* counts were numerically higher compared to the corn-SBM diet, but numerically lower than birds fed BF. Broilers fed corn-SBM resulted in the lowest counts of *C. perfringens*. Overall, barley and fishmeal inclusion increased the incidence of *C. perfringens*, and their combination resulted in the highest exacerbation of *C. perfringens*. Also, the FDT proved to be an effective method to detect the differences in *C. perfringens* counts from the intestines of chickens fed different diets.

Key words: *Clostridium perfringens*, Fung Double Tube, broiler, barley, fishmeal

Introduction

It has been estimated that Necrotic enteritis (NE) costs the poultry industry USD 2 billion a year worldwide (Geier *et al.*, 2010). Necrotic enteritis has been diagnosed in most areas of the world where poultry is raised (Jia *et al*., 2009) and its pathogenesis has been proposed. *Clostridium perfringens*, the causal microorganism, attaches to epithelial lesions most often caused by coccidiosis. This tissue then becomes necrotic due to a combination of factors such as, bacteria proliferation and heterophil lysis. This continued necrosis results in a short, flat villus with reduced nutrient absorption capacity (Long *et al.*, 1974). Necrotic enteritis is exacerbated by predisposing factors including coccidiosis and certain feed ingredients (Hagler, 1986; Yegani and Korver, 2008). The acute symptoms of this disease include depression, inappetence, reluctance to move, diarrhea, and ruffled feathers. Mortality rates can reach up to 50% (Miller, 1998). It has been estimated that the symptomless version of NE, subclinical necrotic enteritis (SNE), costs the poultry industry as much as $0.05 per bird (Hofacre *et al*., 2003). Subclinical necrotic enteritis often remains undiagnosed because there are no obvious signs of disease in the flock; therefore, the disease is untreated. Some of the inconspicuous signs are poor growth performance, wet litter, and possible contamination of food products (Palliyeguru, *et al*., 2010).

The effect of diet composition on NE has been studied throughout the years. The type of cereal grain included in broiler diets has been shown to affect the numbers of *C. perfringens* in the gastrointestinal (GI) tract of chickens. Barley and rye have been shown to contain gums, which hamper digestion in birds (Hagler, 1987). Wheat, rye, barley, and oat groats may have varying levels of complex carbohydrates, including arabinoxylans and beta-glucans, which may interfere with digestion (Riddell and Kong, 1992). *Clostridium perfringens* is a part of the normal flora of broiler chickens GI tract. The activity of this microorganism can be influenced by the presence of viscous polysaccharides associated with poor broiler growth performance. The inclusion of these complex carbohydrates leads to microbial bile acid deconjugation, which can be catalyzed by *C. perfringens*. Engberg *et al.* (2002) compared pellet and mash feeding to determine the influence of feed form on intestinal *C. perfringens*. Results showed that pelleting reduced counts of *C. perfringens* in the GI tract of broiler chickens. The low counts of *C. perfringens* were likely due to decreased amounts of undigested fragments in the small intestine, since pelleting increases feed ingredient digestibility.

Another factor that may play a key role in the severity of NE is both source and inclusion rates of protein ingredients in poultry diets. Truscott and Al-Sheikhly (1977) successfully reproduced NE in chickens by feeding rations containing 50% fishmeal along with a *C. perfringens* challenge. In their preliminary studies, fishmeal was not included in feed formulations, and NE was not consistently produced. Thus, a change in diet formulation was necessary to exacerbate *C. perfringens* concentrations. This was also found by Parish (1971). Presently, NE models often include the addition of fishmeal to poultry rations (Keyburn *et al*., 2008; Williams, 2005). Kaldhusdal and Skejerve (1996) researched the interaction between barley, maize, and wheat, and animal protein rate inclusion. Above-median levels of animal protein resulted in a significant association between barley, wheat, and maize, and disease incidence in the cold season. Drew *et al*. (2004) studied the addition of fishmeal and soy protein concentrate at different levels on the population of *C. perfringens* in the GI tract of broilers. Chickens fed fishmeal diets had significantly higher counts of *C. perfringens* in the ileum and ceca compared to birds fed soy protein concentrate rations. Wilkie *et al*. (2005) studied the inclusion of fishmeal, meat/bone meal, feather meal, corn gluten meal, soy protein concentrate, pea protein concentrate, and potato protein concentrate and numbers of *C. perfringens* in broiler chicken intestines. Birds fed proteins from an animal source, with the exception of potato protein concentrate, resulted in significantly higher counts of *C. perfringens* in chicken intestines. These diets were found to contain the highest concentrations of glycine as compared to proteins from a plant source.

Antibiotics have been shown to be an effective tool to control NE in poultry. Prescott *et al*. (1978) included zinc bacitracin at different concentrations in drinking water after a *C. perfringens* challenge. Levels of 200 and 400 mg/gallon of water were effective in preventing NE in challenged birds. Engberg *et al.* (2000) included zinc bacitracin in broiler diets for six weeks. Broilers fed zinc bacitracin had significantly lower numbers of *C. perfringens* in intestinal contents. George *et al*. (1982) studied the efficacy of different levels (5-40 g/ton) of in-feed virginiamycin in chickens challenged with *C. perfringens*. Broiler chickens fed any level of virginiamycin had significantly lower mortality and lesion scores when compared to the control, challenged chickens. Bolder *et al.* (1999) inoculated broiler chickens with *C. perfringens* and fed rations containing flavophospholipol (FPL). At six weeks, broilers treated with FPL had reduced shedding rates of *C. perfringens* and fewer birds carried the bacterium. Yet another *C.*

perfringens challenge study was performed by Collier *et al*. (2003) to determine the effect of Tylosin in broiler diets on *C. perfringens* colonization. Tylosin addition reduced the concentration of *C. perfringens* in chicken intestines. Furthermore, the prevalence of NE lesions was reduced.

Clostridium perfringens is a spore-forming microorganism, which facilitates its capacity to survive in numerous environments. *Clostridium perfringens* is often found in water, soil, workers clothing, and boots (McDevitt *et al*., 2006; Van Immerseel *et al*., 2004). Pedersen *et al*. (2003) experimentally infected broiler chickens placed in isolator facilities with three strains of *C. perfringens*. Intestinal samples from control, uninoculated birds had counts of *C. perfringens*, demonstrating the ubiquity of this microorganism even after careful attention was paid to control the environment. Craven *et al*. (2001a) studied the prevalence of *C. perfringens* in three commercial broiler hatcheries. Eggshell fragments, chick fluff, and paper pads were sampled to determine the presence of *C. perfringens*. All facilities consistently tested positive for *C. perfringens* over different sampling days, with an overall incidence of 20%. In another study by Craven *et al.,* (2001b) samples analyzed for *C. perfringens* included: paper pads, chicken feces, water line swabs, water cup swabs, litter, feed hoppers, feed, wall drag swabs, fan drag swabs, mice, wild bird feces, feces of other farm animals, insects not on fly strips, soil, standing water, boots, flying insects, cecal droppings, coops before and after transport, scald water, chill water, and chicken carcasses. All samples tested positive during some point of the 6-8 week period. The highest incidences were found in samples obtained from walls, fans, fly strips, dirt outside the house, and boots.

Due to the omnipresence and economical impact of *C. perfringens*, it is of the utmost importance to develop novel methods to detect and enumerate it from different types of samples. Fung (1980) developed the Fung Double Tube (FDT) and it has been used to study *C. perfringens* in food samples. Ali and Fung (1990) studied the occurrence of *C. perfringens* in ground turkey and ground beef using three methods, the FDT, GasPak Anaerobic system, and Oxyrase enzyme. The FDT successfully detected *C. perfringens* in ground turkey and ground beef at a significantly higher rate than the other two methods. Researchers have modified this method for different applications such as, colony PCR confirmation (Ruengwilysup *et al*., 2009), and media supplementation (Sabah and Fung, 2002). Based on previous research performed in our laboratory, the FDT was a superior method to detect and enumerate *C. perfringens* when

compared to anaerobically incubated petri plates. Therefore, the FDT was used to detect and enumerate *C. perfringens* from broiler chickens fed different experimental diets containing *C. perfringens*-exacerbating feed ingredients such as, barley and fishmeal, for 21 days.

Materials and Methods

General Procedures

Animal Care

All broilers were raised following protocols established by the Kansas State University Institution of Animal Care and Use Committee. These experiments were conducted using day-old Cobb 500 (Cobb-Vantress, Siloam Springs, AR) male chicks. For Experiment 1, chicks were housed in floor pens measuring: 1.52 x 1.66 m. In Experiment 2, floor pens measured: 3 x 2.4 m. All birds were raised on clean, pine wood shavings at the Thomas B. Avery Poultry Research Unit (Manhattan, KS). There were 20 chicks per pen, three replicates, and four diet treatments for a total of 240 birds for each experiment. A ziggity nipple water drinking system was set up in each pen and was manually adjusted as birds grew to ensure the watering system was kept at a proper level. Metal hanging feeders were used for the first experiment, and self-feeding feeders (Choretime C3 bottom dispensers) for the second. The chicks were kept under 24 h of light for the first three days, and 23L:1D for the duration of the experiments. Broiler chicks were maintained at 33^{o}C for the first three days and temperature was raised by 2.5^{o}C every seven days until the culmination of the study. For Experiment 1, nine day-old chicks were sampled before farm placement. They were randomly selected and then euthanized by cervical dislocation in order to determine if any *C. perfringens* was already present in the GI tract. For both experiments, broilers were raised from d 0 to 21 with feed and water provided *ad libitum*. Deceased birds were removed as necessary. On d 21, three birds from every pen (n=36 per experiment) were randomly selected and euthanized by cervical dislocation for sampling.

Dietary treatments

For Experiment 1, four rations were formulated: Corn-SBM, Expanded corn, Low CP/High synthetic amino acid, and Barley/fishmeal. Experiment 2 consisted of four diets: Corn-SBM, Barley, Fishmeal, and Barley/fishmeal. All diets were formulated to meet or exceed

nutrient concentrations recommended by the NRC (1994; Tables 3.1 and 3.2). Ground corn used for the expanded corn diet was treated at 180^{o}F and 100 PSI and reground.

Sampling

Broilers were necropsied and the jejunum (bile duct entrance to Meckel's diverticulum) and ileum (Meckel's diverticulum to ileo-cecal junction) were quickly harvested. The jejunum and ileum were cut longitudinally, and along with any chyme, placed in a filtered stomacher bag, weighed, and diluted with 0.1% peptone water (Difco) and stomached (Stomacher®, Seward Laboratory Systems Inc., 1648 Locust Avenue, Bohemia NY) for two minutes. Then, ten-fold serial dilutions were made with 0.1% peptone water (Difco) and plated using the FDT method.

Environmental samples were also obtained at 0 and 21 days for the Experiment 1. Three, 100cm^2 areas from: the entrance of the house, halfway through the house, and in front of the last pen were sampled by sponge swabbing using 0.1% peptone water (Difco) moistened sponges. The same sponge swabbing methodology was employed to sample the walls from three random pens. The trough of three metal hanging feeders was swabbed with a sponge, similar to the floor and walls procedure. Nipples were sampled by sterile swab moistened with 0.1% peptone water (Difco). Feed samples were collected from every diet using a grain probe. One gram of litter and ten grams of every diet were weighed and diluted with peptone water (Difco). In order to obtain air samples, three milliliters of peptone water (Difco) were pipetted into FDT outer tubes and placed in front of a blowing fan for thirty seconds. One mL was plated in duplicate, and the rest serially diluted.

Growth performance data was recorded for Experiment 2. On days 7 and 21, feed and bird weight were recorded per pen. Broiler body weight gain (BWG) and feed conversion (F:G) were calculated using these values. Feed conversion included mortality weight.

Agar Media

Considering previous data, Tryptose Sulfite Cycloserine (TSC) made using Shahidi Ferguson Perfringens (SFP) (Difco) and D-cycloserine (Sigma-Aldrich) is the superior agar for *C. perfringens* detection and enumeration; thus, it was chosen for this set of experiments. Agar base was tempered to 45^{o}C before addition of 0.4 mg of D-cycloserine (Sigma-Aldrich)/L of SFP (Difco) to make TSC.

Fung Double Tube

The FDT were prepared by sterilizing the capped outer tubes (15cm x 1.5cm OD) separately from the inner tubes (15cm x 1.0cm OD). The inner tubes were placed in a rack, wrapped in aluminum foil and sterilized. After autoclaving the media, 25 mL of molten (45^{o}C) agar were pipetted into the outer tube. FDT were kept in water baths at 45^{o}C until inoculation. When samples were ready to be plated, one-mL was inoculated into the FDT. The FDT were inverted three times to distribute the sample in the medium. The inner tube was then aseptically inserted using forceps, thus creating a thin agar layer. Then, the outer tube was capped. The FDT were placed directly in the incubator at 37^{o}C for 24 hours. After incubation, large (~2mm) black colonies were enumerated and recorded.

Confirmation Procedure

After samples were appropriately enumerated and recorded, two black colonies presumptive for *C. perfringens* were picked for confirmation. First, the inner tube was removed exposing the agar layer. Then, a piece of agar containing the desired colony was carefully extracted from the outer tube using disposable, sterile loops. Each colony was streaked for isolation on CDC anaerobe 5% sheep blood agar (BD Diagnostic Systems) and anaerobically incubated for 24 hours. Colonies exhibiting beta-hemolysis were picked and a lawn was streaked on CDC anaerobe 5% sheep blood agar (BD Diagnostic Systems). Plates were anaerobically incubated for 24 hours. These samples were then inoculated into RapID-ANA II kits (Innovative Diagnostic Systems, Inc., Atlanta, Ga.) and results were recorded after four hours of incubation as specified by RapID-ANA II instructions.

Statistical Analysis

The experimental data were analyzed as a completely randomized design. The log values obtained from three replications based on the variable (dietary treatment) were analyzed using the MIXED procedure in SAS (Release 9.1 for Windows, SAS Institute, Cary, NC). Broiler chickens and pens were the experimental units for log values and growth parameters, respectively. Least square means were calculated to separate means based on an alpha of 0.05.

Results

There were no clinical signs of necrotic enteritis observed in the flock during the length of both experiments.

Environmental and chick samples

Broiler intestinal samples and environmental samples were evaluated for the presence of *C. perfringens* at days 0 and 21. In Experiment 1, samples obtained from the air, nipples, and litter at day 0 showed no *C. perfringens* counts. Floor samples for the same testing period resulted in 0.99 log CFU/cm. Wall swabs had 0.63 log CFU/cm of *C. perfringens*. Feeders (2.06 log CFU/mL) had the highest counts of *C. perfringens* of all environmental samples. The corn, synthetic, and barley diets resulted in undetectable levels of *C. perfringens*. The diet containing expanded corn had 2.31 log CFU/g of *C. perfringens*. Intestinal samples from broiler chicks had only 0.54 log CFU/g of *C. perfringens* (Table 3.3). In Experiment 2, *C. perfringens* was not found in the corn diet (Table 3.8). The diets containing barley and fishmeal resulted in 2.13 and 2.93 log CFU/g of *C. perfringens*, respectively. Lastly, rations composed of both barley and fishmeal had 2.41 log CFU/g of *C. perfringens*. After 21 days, environmental counts for Experiment 1 are shown on Table 3.4. Air, nipple, and wall samples from corn, expanded corn, and barley treatments did not contain *C. perfringens*. Floor samples resulted in 1.13 log CFU/cm of *C. perfringens*. The wall sample obtained from a synthetic pen had *C. perfringens* counts of 0.81 log CFU/g. Litter samples collected from the corn, expanded corn, synthetic, and barley treatment resulted in 2.41, 3.17, 2.96, and 2.29 log CFU/g, respectively. Samples from feeders for diet treatments, corn, expanded, synthetic, and barley were as follows: 0.98, 0.56, 0.20, and 0.98 log CFU/g, respectively. Lastly, *C. perfringens* counts for the remaining feed were obtained and corn had 2.37 log CFU/g. Expanded corn resulted in 1.70 log CFU/g. The diets formulated with high amounts of synthetic amino acids, and the ration with barley had 0.48 and 0.40 log CFU/g, respectively.

Intestinal samples

After 21 days, broiler chicken intestinal samples were analyzed for *C. perfringens* content. In Experiment 1 (Table 3.5), the lowest concentration of *C. perfringens* was found in birds fed the expanded corn diet, averaging 3.64 log CFU/g. Chickens provided with the corn and high synthetic AA rations resulted in 3.89 log CFU/g. Broiler chickens fed the barley diet

had significantly higher (P<0.05) concentrations of *C. perfringens* compared to the other three dietary treatments. In Experiment 2 (Table 3.9), birds fed the corn ration resulted in the lowest (P<0.05) counts (2.70 log CFU/g) of *C. perfringens*. Broiler chickens that consumed the barley ration and the fishmeal ration resulted in 3.58 log CFU/g, which is numerically higher than the corn treatment. Chickens fed the barley/fishmeal ration resulted in *C. perfringens* counts of 4.15 log CFU/g; numerically higher than barley and fishmeal alone, and significantly (P<0.05) higher than the corn treatment.

Growth parameters

For Experiment 2, body weight gain (BWG) and feed conversion (F:G) were determined and results are displayed in Tables 3.6 and 3.7, respectively. Birds fed the corn diet weighed 0.792 kg, significantly (P<0.05) less than all other treatments. Broiler chickens supplied with the barley ration weighed an average of 0.816 kg, which is significantly (P<0.05) lower than birds fed fishmeal or a combination of barley/fishmeal. Chickens sampled from the barley/fishmeal treatment were significantly (P<0.05) heavier (0.883 kg) than all other dietary treatments. Birds fed the fishmeal diet weighed 0.841 kg, which is significantly (P<0.05) higher than corn and barley, and significantly (P<0.05) lower than the barley/fishmeal combination. Chickens fed barley/fishmeal had the most efficient feed conversion, 1.06, significantly (P<0.05) lower than the barley and corn diets. Corn (1.11) had the poorest feed conversion, which was significantly (P<0.05) different from birds fed fishmeal or barley/fishmeal. Feed efficiencies for the barley, and fishmeal diets were 1.09 and 1.08, respectively.

Confirmation

Results obtained through the confirmation procedure with RapID-ANA II kits for environmental and bird samples at day 0 are shown on Table 3.10. The rapid kit identified (No. of samples): *Clostridium perfringens, C. sporogenes, C. innocuum*, *C. hastiforme*, and *C. tertium* from floor samples. In wall samples, *C. tetani*, and *C. perfringens* (3) were found. Feeder samples resulted in *C. perfringens*, *C. butyricum*, and *L. minutis*. As far as feed samples, *C. perfringens* (4) was found in all diets, except barley. *Clostridium innocuum* was also identified in expanded corn. Broiler chick intestinal samples were identified as *C. innocuum* (3), and *L. acidophilus*. On day 0, 28 samples did not code for identification. Confirmation results for environmental samples after 21 days are shown in Table 3.11. In the expanded corn treatment,

samples from the feed and feeder were identified as *C. innocuum* and *C. perfringens*, respectively. No identifications were made for the wall and litter samples. In the corn treatment, *C. innocuum* was found in the litter and feeder. The litter sample was also positive for *L. acidophilus*. In the synthetic AA treatment, the feed sample was positive for *C. innocuum. Eubacterium aerofaciens* was found in the feeder, and a wall sample was identified as *C. hastiforme*. In the barley treatment, the feed was confirmed positive for *C. innocuum*, which was also found in the feeder, along with *C. perfringens*. Floor samples were positive for *E. aerofaciens, L. acidophilus*, and *C. innocuum*. A total of 19 environmental samples for day 21 did not code for identification. Lastly, identifications of broiler chicken intestinal samples for Experiment 1 are shown in Table 3.12. A total of 37 samples were not identified by the rapid kit. In the corn diet, *C. perfringens, bifidobacterium, C. subterminale*, and *L. acidophilus* (4) were found. While *C. perfringens* (2), and *L. acidophilus* (3) were isolated from chickens fed expanded corn. Chickens in the synthetic AA ration were positive for *C. perfringens* (2), *A. mayeri, C. butyricum, C. subterminale, C. tertium* (2), and *L. acidophilus* (4). Samples from birds fed the barley ration were identified as *C. perfringens, bifidobacterium* (3), *L. acidophilus* (6), and *L. fermentum*.

In Experiment 2, confirmation results for diet samples are found in Table 3.13. Identifications were not possible for a total of five samples. *Clostridium tetani* was found in the fishmeal diet. The barley/fishmeal combination was positive for *C. innocuum, L. casei,* and *L. acidophilus*. Broiler chicken intestinal samples identification results are displayed on Table 3.14. Birds fed diets with corn were positive for *C. perfringens, C. subterminale*, and *E. limosum*. Samples of broiler chickens fed fishmeal were identified as *C. clostridioforme*, and *L. acidophilus*. Lastly, the barley/fishmeal ration resulted in *C. innocuum*, and *C. perfringens* identifications. A total of 70 samples did not code for identification.

Discussion

Clostridium perfringens is a very resilient microorganism due to its ability to produce spores. In our first experiment, swab samples obtained from feeders, pen walls, and floors were contaminated with *C. perfringens* at the beginning of the trial, as well as, after 21 days. These results resonate with the findings of Craven *et al.,* (2001b). In their study, 53% of wall swab samples from 138 farms tested positive for the presence of *C. perfringens*, although

enumerations were not performed. Our methodology did not return *C. perfringens* counts for air and nipple drinker samples. This may be related to the sampling procedure. Cotton swabs were used to collect samples from nipple drinkers, and the small surface area of the swab combined with the bacterial attachment that must happen in order to obtain counts of *C. perfringens* may have jeopardized accurate sampling. As far as air samples, a longer sampling time should be implemented to evaluate *C. perfringens* counts. Samples of the corn rations fed in Experiment 1 and 2 did not show colonies of *C. perfringens*. This finding may be of interest since some researchers have contemplated that digested corn may contain certain anti-*Clostrdium* substances (Van Immerseel *et al*., 2004). Broiler diets formulated with barley, fishmeal, and BF in Experiment 2, had approximately 2.5 log CFU/g of *C. perfringens*, resulting in a viable contamination source. The diet containing expanded corn was the only one with positive counts of *C. perfringens* in Experiment 1. This may be explained by the additional processing required to expand corn. *Clostridium perfringens* spores are resistant to the conditions encountered in an expander, thus this processing would provide more contact surfaces opportunities with which the feed may become contaminated with spores of *C. perfringens*.

The effect of diet on the incidence of NE has been previously studied. In Experiment 1, the diet with highest concentration of *C. perfringens* contained barley and fishmeal. Both of these feed ingredients have been shown to exacerbate counts of *C. perfringens* in the GI tract of broilers. Therefore, Experiment 2 was designed to better understand if barley and fishmeal alone would increase *C. perfringens* counts, and if a cumulative effect existed. As other researchers have found, fishmeal and barley alone increased *C. perfringens* enumerations in broiler chicken intestines. Furthermore, our results indicate that a synergistic effect was found, since the highest concentrations of *C. perfringens* were found in the BF diet.

Researchers have investigated the influence of protein source and cereal grains on *C. perfringens*. Wilkie *et al*. (2005) found that birds fed proteins of animal source, such as fishmeal, had increased counts of *C. perfringens*. Similar results have been documented (Truscott and Al-Sheikhly, 1977; Drew *et al*., 2004). Yet another protein source implicated in exacerbating *C. perfringens* counts is potato concentrate (Wilkie *et al*., 2005; Palliyeguru *et al*., 2010). *Clostridium perfringens* has been shown to thrive in feed ingredients high in methionine and glycine (Wilkie *et al*., 2005; Williams, 2005). Since fishmeal has been found to increase counts of *C. perfringens*, numerous researchers have formulated diets containing up to 50% fishmeal in

the development process of a NE reproduction model (Prescott, 1979; George *et al*., 1982, Keyburn *et al*., 2008). Hofshagen and Kaldhusdal (1992) found higher concentrations of *C. perfringens* in diets formulated with barley instead of corn. A significant difference could not be established because oats and wheat were included in all diets, which could have confounded the results. Riddell and Kong (1992) determined that diets containing barley produced mortality rates similar to those found in birds fed wheat, which were significantly higher than chickens fed corn when birds were challenged with *C. perfringens*. Several authors have implicated barley as a promoter of *C. perfringens* in poultry and have reasoned that it may be due to its high content of non-starch polysaccharides (NSP). Increased amounts of NSP results in increased gut viscosity, which in turns increases gut stasis. This provides additional time for *C. perfringens* cells to attach to intestinal epithelial cells where lesions are eventually formed (Bedford, 1996; Kaldhusdal and Skjerve, 1996; Branton *et al*., 1997; Pedersen *et al.*, 2007; Jia *et al*., 2009).

As far as dietary antimicrobial effects, *C. perfringens* counts were similar in Experiment 1 and 2, suggesting that a more complicated interaction is taking place in the GI tract of broiler chickens. This is in agreement with Engberg *et al*. (2000), establishing that antibiotics such as, zinc bacitracin and salinomycin do not eradicate *C. perfringens* from the GI tract. This further emphasizes the need for a multifactorial approach to the NE problem in poultry flocks.

The confirmation procedure chosen for these experiments was the RapID-ANA II. Previous food product *C. perfringens* inoculation studies in our laboratory had successfully included the RapID-ANA II kit as a confirmation method for black *C. perfringens* colonies. In Experiments 1 and 2, *C. perfringens* could not be consistently confirmed using the RapID-ANA II kit. The confirmation results could have been negatively affected by: enzyme production, and database updating. Ruengwilysup *et al*. (2008) established that confirmation procedures that necessitate enzyme production might result in biased or inaccurate readings. Bacteria are unpredictable, at times they may not multiply as fast as expected, resulting in a limited amount of colonies which will not produce enough enzymes to breakdown biochemicals in rapid kits such as, RapID-ANA II. Moreover, even if enough bacterial cells are present, it is possible that they do not produce the enzyme or the enzymes produced are weak. This is a major concern since our experiments were performed without a *C. perfringens* challenge; thus, we could not control the type of *C. perfringens* in chicken intestinal samples. Also, the RapID-ANA II kit depends on a digital database to interpret codes. If this database is not updated on a regular basis, keeping in

mind that the generation time for some *C. perfringens* strains is as short as eight minutes, the database may consider a wild-type *C. perfringens* strain as unidentifiable.

In conclusion, *C. perfringens* concentrations are increased in broiler chickens fed diets containing barley, and fishmeal, as compared to corn formulated rations. Additionally, the combination of barley and fishmeal resulted in an additive effect. Since antibiotics alone may not always successfully control *C. perfringens*, and have the potential for subtherapeutic use loss in the US, a multifactorial approach must be considered and investigated.

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Table 3.1 Experiment 1 – Diet formulations and nutrient composition (%, as-fed basis)

Ingredients	Corn-SBM	Expanded corn	Synth AA	Barley & fishmeal
Corn	57.51	57.51	65.50	
Barley				56.38
Soybean meal (48%)	32.99	32.99	28.78	29.27
Porcine meat and bone meal (47.9%)	4.00	4.00		
Menhaden fishmeal				4.00
Soy oil	3.04	3.04	2.02	7.39
Limestone	0.66	0.66	0.83	0.53
Defluorinated phosphate	0.97	0.97	1.86	1.61
Salt	0.28	0.28	0.23	0.17
L-Lysine			0.10	
L-Threonine			0.07	
L-Valine				0.15
DL-Methionine	0.19	0.19	0.28	0.17
Feed additives[123]	0.35	0.35	0.35	0.35
Calculated Composition				
Metabolizable energy (Kcal/kg)	3200	3200	3200	3200
Crude protein	23.00	23.00	19.83	23.00
Lysine	1.23	1.23	1.10	1.27
Methionine	0.54	0.54	0.58	0.53
Tryptophan	0.29	0.29	0.25	0.32
Threonine	0.85	0.85	0.80	0.85
Calcium	1.00	1.00	1.00	1.00
Available phosphorus	0.45	0.45	0.45	0.45
Sodium	0.20	0.20	0.20	0.20

[1] Supplied per kg of diet: manganese, 0.02%; zinc, 0.02%; iron, 0.01%; copper, 0.0025%; iodine, 0.0003%; selenium, 0.00003%; folic acid, 0.69 mg; choline, 386 mg; riboflavin, 6.61 mg; biotin, 0.03 mg; vitamin B6, 1.38 mg; niacin, 27.56 mg; pantothenic acid, 6.61 mg; thiamine, 2.20 mg; menadione, 0.83 mg; vitamin B12, 0.01 mg; vitamin E, 16.53 IU; vitamin D3, 2,133 ICU; vitamin A, 7,716 IU.

[2] Monensin 0.099 g/kg, Elanco Animal Health, Indianapolis, IN.

[3] Bacitracin methylene disalicylate. 0.055 g/kg, Alpharma, Bridgewater, NJ.

Table 3.2 Experiment 2 – Diet formulations and nutrient composition (%, as-fed basis)

Ingredients	Corn-SBM	Barley	Fishmeal	Barley & fishmeal
Corn	53.30	45.40	56.90	49.50
Barley		7.46		7.46
Soybean meal (48%)	38.80	38.6	33.20	32.60
Menhaden fishmeal			4.00	4.00
Soy oil	3.63	4.29	2.50	3.10
Limestone	1.72	1.73	1.41	1.42
Defluorinated phosphate	1.53	1.51	1.04	1.01
Salt	0.46	0.47	0.46	0.47
L-Lysine	0.04	0.04	0.01	0.02
DL-Methionine	0.25	0.26	0.21	0.23
Feed additive[1]	0.25	0.25	0.25	0.25
Calculated Composition				
Metabolizable energy (Kcal/kg)	3070	3070	3070	3070
Crude protein	22.90	23.00	22.90	22.90
Lysine	1.32	1.32	1.32	1.32
Methionine	0.60	0.60	0.60	0.60
Tryptophan	0.32	0.32	0.30	0.31
Threonine	0.88	0.88	0.88	0.88
Calcium	1.00	1.00	1.00	1.00
Available phosphorus	0.45	0.45	0.45	0.45
Sodium	0.20	0.20	0.20	0.20

[1] Supplied per kg of diet: manganese, 0.02%; zinc, 0.02%; iron, 0.01%; copper, 0.0025%; iodine, 0.0003%; selenium, 0.00003%; folic acid, 0.69 mg; choline, 386 mg; riboflavin, 6.61 mg; biotin, 0.03 mg; vitamin B6, 1.38 mg; niacin, 27.56 mg; pantothenic acid, 6.61 mg; thiamine, 2.20 mg; menadione, 0.83 mg; vitamin B12, 0.01 mg; vitamin E, 16.53 IU; vitamin D3, 2,133 ICU; vitamin A, 7,716 IU.

Table 3.3 Experiment 1 – *C. perfringens* counts in environmental and chick samples, day 0

Sample (No. of samples)	Avg. log CFU/g
Floor	0.99
Air	0
Nipples	0
Walls	0.63
Litter	0
Feeders	2.06
Exp	2.31
Corn	0
Synthetic	0
Barley	0
Broiler chicks	0.54

Nipples, walls, litter, and feeder samples collected from three random pens previous to bird placement.

Table 3.4 Experiment 1 – *C. perfringens* counts in environmental samples, day 21

Sample (No. of samples)	Avg. log CFU/g
Floor	1.13
Air	0
Nipples	0
Wall – Corn	0
Wall – Exp	0
Wall – Synthetic	0.81
Wall – Barley	0
Litter – Corn	2.41
Litter – Exp	3.17
Litter – Synthetic	2.96
Litter – Barley	2.29
Feeder – Corn	0.98
Feeder – Exp	0.56
Feeder – Synthetic	0.20
Feeder – Barley	0.98
Exp	1.70
Corn	2.37
Synthetic	0.48
Barley	0.40

Table 3.5 Experiment 1 – *C. perfringens* counts in broiler intestinal samples, day 21

Diet	Avg. log CFU/g
Corn	3.89
Expanded corn	3.64
Synthetic	3.89
Barley	5.96*

* Means within rows with no common superscripts differ significantly (P<0.05).

Table 3.6 Experiment 2 – Body Weight Gain, days 7 to 21

Diet	kg
Corn	0.79[d]
Barley	0.82[c]
Fishmeal	0.84[b]
Barley/fishmeal	0.88[a]

[a,b,c,d] Means within rows with no common superscripts differ significantly (P<0.05).

Table 3.7 Experiment 2 – Feed:Gain, days 7 to 21

Diet	(kg:kg)
Corn	1.11[c]
Barley	1.09[bc]
Fishmeal	1.08[ab]
Barley/fishmeal	1.06[a]

[a,b,c] Means within rows with no common superscripts differ significantly (P<0.05).

Table 3.8 Experiment 2 – *C. perfringens* counts in diet samples, day 0

Diet	Avg. log CFU/g
Corn	0
Barley	2.13
Fishmeal	2.93
Barley/fishmeal	2.41

Table 3.9 Experiment 2 – *C. perfringens* counts in broiler intestinal samples, day 21

Diet	Avg. log CFU/g
Corn	2.70[b]
Barley	3.58[a,b]
Fishmeal	3.58[a,b]
Barley/fishmeal	4.15[a]

[a,b] Means within rows with no common superscripts differ significantly (P<0.05).

Table 3.10 Experiment 1 – Environmental and chick samples confirmation results, day 0

Sample	Unidentified	Isolates (No. of samples)
Floor	4	*C. perfringens, C. sporogenes, C. innocuum C. hastiforme, C. tertium*
Walls	6	*C. tetani, C. perfringens* (3)
Feeders	4	*C. perfringens, C. butyricum, L. minutis* (2)
Expanded corn	2	*C. innocuum, C. perfringens* (2)
Corn	3	*C. perfringens*
Synthetic	2	*C. perfringens*
Barley	4	
Birds	3	*C. innocuum* (3)*, L. acidophilus*

Table 3.11 Experiment 1 – Environmental samples confirmation results, day 21

	Sample	Unidentified	Isolates
Exp	**Litter**	2	
	Feed	1	*C. innocuum*
	Feeder	1	*C. perfringens*
	Wall	2	
Corn	**Litter**		*C. innocuum, L. acidophilus*
	Feed	2	
	Feeder	1	*C. innocuum*
Synthetic	**Feed**	1	*C. innocuum*
	Feeder	2	*E. aerofaciens*
	Wall	1	*C. hastiforme*
Barley	**Feed**	1	*C. innocuum*
	Feeder	2	*C. perfringens, C. innocuum*
Floor	**Entrance**	1	*E. aerofaciens*
	Midway	1	*L. acidophilus*
	End	1	*C. innocuum*

Table 3.12 Experiment 1 – Broiler intestinal samples confirmation results, day 21

Diet	Unidentified	Isolates (No. of samples)
Corn	11	*Bifidobacerium, C. perfringens, C. subterminale, L. acidophilus* (4)
Exp	12	*C. perfringens* (2), *L. acidophilus* (3)
Synthetic	7	*A. mayeri*, *C. butyricum, C. perfringens* (2), *C. subterminale*, *C. tertium* (2), *L. acidophilus* (4)
Barley	7	*Bifidobacerium* (3), *C. perfringens, L. acidophilus* (6), *L. fermentum*

Table 3.13 Experiment 2 – Diet samples confirmation results, day 0

Diet	Unidentified	Isolates
Fish	2	*C. tetani*
Barley/fishmeal	1	*C. innocuum, L. casei, L. acidophilus*
Fishmeal	2	

Table 3.14 Experiment 2 – Broiler intestinal samples confirmation results, day 21

Diet	Unidentified	Isolates
Corn	14	*C. perfringens, C. subterminale, E. limosum*
Barley	19	
Fishmeal	21	*C. clostridioforme*, *L. acidophilus*
Barley/fishmeal	16	*C. innocuum*, *C. perfringens*

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