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Research Article
The Effect of Altering Feed Formula, Processing and Supplements on *Clostridium* spp. In Broilers Using the Fung Double Tube Method

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Abstract

Background and Objective: Necrotic enteritis (NE) is an acute intestinal infection that severely affects broiler performance and mortality. A new method for improved assay to enumerate Clostridium ssp. enables investigations to focus on how this disease affects broiler flocks. The objective of this study was to evaluate the Fung Double Tube (FDT) method to enumerate *Clostridium* spp. in poultry samples from the environment and feed rations. **Materials and Methods:** Twenty chicks were placed in floor pens with shavings, with 3 replications per diet and 240 per trial. Diets in Trial 1 were: Corn-soybean meal (CS), Expanded corn, Low-crude protein (19.8%)/High synthetic amino acids (SAA) and Barley (56%)-fishmeal (4%, BF) and contained an antibiotic and a coccidiostat. Diets in Trial 2 were: CS, Barley (7.5%), Fishmeal (4%) and BF and no antibiotic or coccidiostat. In Trial 1, environmental samples were collected while feed samples were analyzed in Trial 2. **Results:** In Trial 1, birds fed BF had the highest ($p<0.05$) counts (5.96 $\log_{10}$ CFU g^{-1}) of *Clostridium* spp. *Clostridium* spp. was isolated from feed, feeders, pen walls, floors and litter. In Trial 2, birds fed the CS diet (2.70 $\log_{10}$ CFU g^{-1}) had lower ($p<0.05$) counts than birds fed BF (4.15 $\log_{10}$ CFU g^{-1}). Broilers fed fishmeal (3.58 $\log_{10}$ CFU g^{-1}) and barley (3.58 $\log_{10}$ CFU g^{-1}) had *Clostridium* spp. counts that numerically higher compared to the CS diet but lower than birds fed BF. **Conclusion:** The FDT method is useful to follow NE levels in poultry, in the feed and their environments.

Key words: Broiler, *Clostridium* spp., feed formulas, feed processing, fung double tube

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

It has been estimated that Necrotic Enteritis (NE) costs the poultry industry USD 2 billion a year worldwide[1]. Mild NE in broilers was shown to reduce body weight gain by 12% and FCR by 10.9%[2]. Necrotic enteritis has been diagnosed in most areas of the world where poultry is raised[3] and its pathogenesis has been proposed. *Clostridium perfringens*, the causal microorganism, attaches to intestinal 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 short, flat villi with reduced nutrient absorption capacity[4]. Necrotic enteritis is exacerbated by predisposing factors that include coccidiosis and certain feed ingredients[5,6]. The acute symptoms of this disease are depression, inappetence, reluctance to move, diarrhea, ruffled feathers and mortality rates can reach up to 50%[7]. 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[8]. In most cases, SNE often remains undiagnosed and untreated because there are no obvious signs of disease in the flock. Some of the inconspicuous signs are: poor growth performance, wet litter and possible contamination of food products[9].

The effect of diet composition on NE has been studied throughout the years. The type of cereal grain included in broiler diets has shown to affect the numbers of *C. perfringens* in the gastrointestinal (GI) tract of chickens. Barley and rye contain gums, which hamper digestion in birds[5]. Wheat, rye, barley and oat groats may have varying levels of complex carbohydrates, including arabinoxylans and beta-glucans, which may interfere with digestion[10]. *Clostridium perfringens* is a part of the normal flora of broiler chickens GI tract[6]. 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.*[11] 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. Broilers fed feeds manufactured to contain lower levels of microorganisms were shown to be less affected by a Clostridia challenge[12].

Another factor that may play a key role in the severity of NE is source and inclusion rates of protein ingredients in poultry diets. Truscott and Al-Sheikhly[13] 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 reproduced. Thus, a change in diet formulation was necessary to exacerbate *C. perfringens* concentrations. This was also found by Parish[14]. Presently, NE models often include the addition of fishmeal to poultry rations[15,16]. Kaldhusdal and Skejerve[17] researched the interaction between barley, maize and wheat and animal protein 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.*[18] 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.*[19] 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.

Antibiotics have been shown to be an effective tool to control NE in poultry. Prescott *et al.*[20] included zinc bacitracin at different concentrations in drinking water after a *C. perfringens* challenge. Levels of 200 and 400 mg gallon^{-1} of water were effective in preventing NE in challenged birds. Engberg *et al.*[11] 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.*[21] studied the efficacy of different levels (5-40 g t^{-1}) 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.*[22] inoculated broiler chickens with *C. perfringens* and fed rations containing flavophospholipol. At six weeks, broilers treated with flavophospholipol had reduced shedding rates of *C. perfringens* and fewer birds carried the bacterium. Another *C. perfringens* challenge study performed by Collier *et al.*[23] to determine the effect of tylosin in broiler diets on *C. perfringens* colonization indicated that 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 that has the ability to survive in numerous environments. *Clostridium perfringens* is often found in water, soil, workers clothing and boots[24,25]. Pedersen *et al*.[26] studied 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*.[27] studied the prevalence of *C. perfringens* in three commercial broiler hatcheries where 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*.[28], 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 and Lee[29] developed the Fung Double Tube (FDT) and it has been used to study *C. perfringens* in food and water[30] samples. Ali and Fung[31] studied the occurrence of *C. perfringens* in ground turkey and ground beef using three methods: 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. Other researchers have further modified the FDT to allow for improved *C. perfringens* detection[32]. Most recently, Barrios *et al*.[33] established the FDT as a superior method to detect and enumerate *Clostridium* spp. when compared to anaerobically incubated petri plates using different media. Therefore, the objective of the this study was to evaluate the applicability of the FDT as a method to enumerate *Clostridium* spp. in poultry feed, litter, environmental swabs and air samples as well as chickens fed different diets without a clostridial challenge to avoid unnecessary animal stress.

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 Trial 1, chicks were housed in floor pens measuring: 1.52×1.66 m. In Trial 2, floor pens measured: 3×2.4 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, 3 replicates and 4 diet treatments for a total of 240 birds for each experiment. A nipple water drinking system was set up in each pen and 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 (Chore time C3 bottom dispensers) for the second. Chicks were kept under 24 h of light for the first 3 days and 23L:1D for the duration of the experiments. Broiler chicks were maintained at 33°C for the first 3 days and temperature was lowered by 2.5°C every 7 days until the culmination of the study. For Trial 1, 9 day-old chicks were sampled before farm placement. They were randomly selected and euthanized by cervical dislocation in order to determine if any *Clostridium* spp. was already present in the GI tract. For both experiments, broilers were raised from day 0-21 with feed and water provided *ad libitum*. Deceased birds were removed as necessary. On d 21, 3 birds from every pen (n = 36 per experiment) were randomly selected and euthanized by cervical dislocation for sampling.

Dietary treatments: For Trial 1, 4 rations were formulated: Corn-SBM (CS), Expanded corn, Low CP/High synthetic amino acid (SAA) and Barley/fishmeal (BF) (Table 1). Trial 2 consisted of 4 diets: CS, Barley, Fishmeal and BF (Table 2). All diets were formulated to meet or exceed nutrient concentrations recommended by the NRC[34]. Ground corn used in the expanded corn diet was treated at 180°F and 100 PSI and reground.

Fung double tube: The FDT were prepared by sterilizing the capped outer tubes (15×1.5 cm OD; Kimax 45066; Fisher Scientific, Hampton, NH) separately from the inner tubes (15×1.0 cm OD; Kimax 450421). The inner tubes were placed in a rack, wrapped in aluminum foil and sterilized. After autoclaving the agar media, 25 mL of molten (45°C) TSC were pipetted into the outer tubes. FDT were kept in water baths at 45°C until inoculation.

Agar media: Considering previous data[33,35], Tryptose Sulfite Cycloserine (TSC) made using Shahidi Ferguson Perfringens (SFP; Difco, Franklin Lakes, NJ) and D-cycloserine (Sigma-Aldrich, St. Louis, MO) is a superior agar for *C. perfringens* detection and enumeration; thus, it was chosen for this set of experiments. Agar base was tempered to 45°C before addition of 0.4 mg of D-cycloserine (Sigma-Aldrich, St. Louis, MO)/L of SFP (Difco, Franklin Lakes, NJ) to make TSC.

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, suspended in sterile 0.1% peptone water (Difco, Franklin Lakes, NJ) and stomached (Stomacher®, Seward Laboratory Systems Inc., Bohemia, NY) for 2 min. Serial dilutions were then made with 0.1% peptone water (Difco, Franklin Lakes, NJ) and1 mL of each sample was inoculated into the FDT and immediately inverted 3 times to distribute the sample in the medium. The inner tube was then aseptically inserted using forceps, thus creating a thin agar layer and the outer tube was capped. The FDT were placed directly in a conventional incubator at 37°C for 24 h. After incubation, large (~2 mm) black colonies presumptive for *C. perfringens* were enumerated and reported as $\log_{10}$ colony-forming units (CFU) per gram of intestinal sample.

Environmental samples were also obtained at 0 and 21 day for Trial 1. Three, 100 cm^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, Franklin Lakes, NJ) moistened sponges. The same sponge swabbing methodology was employed to sample the walls from 3 random pens. The trough of 3 metal hanging feeders was swabbed with a sponge, similar to the floor and walls procedure. Nipple drinkers were sampled by sterile swab moistened with 0.1% peptone water (Difco, Franklin Lakes, NJ). Feed samples were collected from every diet using a grain probe. One gram of litter and 10 g of every diet were weighed and serially diluted with peptone water (Difco, Franklin Lakes, NJ). In order to obtain air samples, 3 mL of peptone water (Difco, Franklin Lakes, NJ) were pipetted into FDT outer tubes and placed in front of a blowing fan for 30 s. One mL was plated in duplicate and the rest serially diluted.

Growth performance data was collected in Trial 2. On days 7 and 21, feed and bird weight were recorded per pen. Individual broiler body weight gain (BWG) and feed conversion (F:G) were calculated using these values. Feed conversion was calculated taking into account mortality weights.

Confirmation procedure: After samples were appropriately enumerated and recorded, black colonies presumptive for *C. perfringens* were picked for confirmation. First, the inner tube was removed exposing the agar layer. 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 Centers for Disease Control and Prevention[36] anaerobe 5% sheep blood agar (BD Diagnostic Systems, Franklin Lakes, NJ) and anaerobically incubated at 37°C for 24 h. Colonies exhibiting beta-hemolysis were picked and a lawn was streaked on CDC anaerobe 5% sheep blood agar (BD Diagnostic Systems, Franklin Lakes, NJ). Plates were anaerobically incubated at 37°C for 24 h. These samples were then inoculated into RapID-ANA II kits (Innovative Diagnostic Systems, Inc., Atlanta, Ga.) and results were recorded after 4 h of incubation at 37°C as per RapID-ANA II user instructions.

Statistical analysis: The experimental data was analyzed using a completely randomized design. The $\log_{10}$ values obtained from 3 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 $\log_{10}$ values and growth parameters, respectively. Least square means were calculated to separate means based on an alpha of 0.05.

RESULTS

Environmental and chick samples: Broiler intestinal samples and environmental samples were evaluated for the presence of *Clostridium* spp. at days 0 and 21 (Table 3). In Trial 1, at day 0, samples obtained from the air, nipples and litter showed no *Clostridium* spp. counts. Floor samples for the same testing period resulted in 0.99 $\log_{10}$ CFU cm^{-1}. Wall swabs had 0.63 $\log_{10}$ CFU cm^{-1} of *Clostridium* spp. Feeders had the highest counts (2.06 $\log_{10}$ CFU mL^{-1}) of *Clostridium* spp. of all environmental samples. The CS, SAA and BF diets resulted in undetectable levels of *Clostridium* spp. The diet containing expanded corn had 2.31 $\log_{10}$ CFU g^{-1} of *Clostridium* spp. Intestinal samples from broiler chicks had only 0.54 $\log_{10}$ CFU g^{-1} of *Clostridium* spp.

In Trial 2, *Clostridium* spp. was not found in the CS diet (Table 3). The diets containing barley and fishmeal resulted in 2.13 and 2.93 $\log_{10}$ CFU/g of *Clostridium* spp., respectively. Lastly, rations composed of both barley and fishmeal had 2.41 $\log_{10}$ CFU g^{-1} of *Clostridium* spp. After 21 days, environmental counts for Trial 1 are shown on Table 3. Air, nipple and wall samples from CS, expanded corn and BF treatments did not contain *Clostridium* spp. Floor samples resulted in 1.13 $\log_{10}$ CFU cm^{-1} of *Clostridium* spp. The wall

Table 1: Trial 1: Diet formulations and nutrient composition (%, as-fed basis)

Ingredients	Corn-SBM	Expanded corn	Synth AA	Barley and 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^{-1})	3200.00	3200.00	3200.00	3200.00
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^{-1}, Elanco Animal Health, Indianapolis, IN. [3]Bacitracin methylene disalicylate. 0.055 g kg^{-1}, Alpharma, Bridgewater, NJ

Table 2: Trial 2: Diet formulations and nutrient composition (%, as-fed basis)

Ingredients	Corn-SBM	Barley	Fishmeal	Barley and 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^{-1})	3070.00	3070.00	3070.00	3070.00
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

sample obtained from a SAA pen had *Clostridium* spp. counts of 0.81 $\log_{10}$ CFU g^{-1}. Litter samples collected from the CS, expanded corn, SAA and BF treatment resulted in 2.41, 3.17, 2.96 and 2.29 $\log_{10}$ CFU g^{-1}, respectively. Samples from feeders for diet treatments, CS, expanded, SAA and BF were as follows: 0.98, 0.56, 0.20 and 0.98 $\log_{10}$ CFU g^{-1}, respectively. Lastly, *Clostridium* spp. counts for the remaining feed were obtained and CS had 2.37 $\log_{10}$ CFU g^{-1}. Expanded corn resulted in

Table 3: Trial 1: *Clostridium* spp. counts (Avg. $\log_{10}$ CFU g^{-1}) using the Fung Double Tube in environmental samples, feed samples and intestinal samples of broilers fed different dietary ingredients in broilers fed different rations, day 0 and day 21

		Day 21				
Sample	Day 0[†]	CS	Expanded	SAA	BF	SEM
Walls	0.63	0	0	0.81	0	
Litter	0.00	2.41	3.17	2.96	2.29	
Feeders	2.06	0.98	0.56	0.20	0.98	
Feed (day 0)		0.00	2.31	0.00	0.00	
Feed (day 21)		2.37	1.70	0.48	0.40	
Intestines	0.54	3.89	3.64	3.89	5.96*	0.28
Air	0.00	0.00				
Nipples	0.00	0.00				
Floor	0.99	1.13				

*Intestines means within columns with no common superscripts differ significantly ($p<0.05$), [†]Nipples, walls, litter and feeder samples collected from three random pens previous to bird placement, [1]Treatment diets; Expanded: Expanded corn, CS: Corn-SBM, SAA: Low CP/high synthetic AA, BF: Barley/fishmeal, [2]Floor samples were obtained from: the entrance of the house, halfway through the house and in front of the last pen. Air and nipple drinkers samples were collected from three random pens

Table 4: Trial 2: Effect of dietary ingredients on broiler individual body weight gain and feed: gain and *Clostridium* spp. counts using the fung double tube with rations and broiler intestines, days 0-21

			Avg. $\log_{10}$ CFU g^{-1}	
	Days 7-21			
			Day 0	Day 21
Diet	kg	kg : kg	Feed	Intestines
CS	0.7900[d]	1.110[c]	0.00	2.70[b]
Barley	0.8200[c]	1.0900[bc]	2.13	3.58[a,b]
Fishmeal	0.8400[b]	1.0800[ab]	2.93	3.58[a,b]
BF	0.8800[a]	1.0600[a]	2.41	4.15[a]
SEM	0.0057	0.0079		0.37

[a-d]Means within rows with no common superscripts differ significantly ($p<0.05$), [1]Treatment diets; CS: Corn-SBM, BF: Barley/fishmeal

1.70 $\log_{10}$ CFU g^{-1}. The diets formulated with high amounts of synthetic amino acids and the ration with barley and fishmeal had 0.48 and 0.40 $\log_{10}$ CFU g^{-1}, respectively.

Intestinal samples: There were no clinical signs of necrotic enteritis observed in the flock during the experiments. After 21 days, broiler chicken intestinal samples were analyzed for *Clostridium* spp. content. In Trial 1 (Table 3), the lowest concentration of *Clostridium* spp. was found in birds fed the expanded corn diet, averaging 3.64 $\log_{10}$ CFU g^{-1}. Chickens provided with the CS and SAA rations resulted in 3.89 $\log_{10}$ CFU g^{-1}. Broiler chickens fed the BF diet had significantly higher ($p<0.05$) concentrations of *Clostridium* spp. compared to the other three dietary treatments. In Trial 2 (Table 4), birds fed the CS ration resulted in the lowest ($p<0.05$) counts (2.70 $\log_{10}$ CFU g^{-1}) of *Clostridium* spp. Broiler chickens that consumed the barley ration and the fishmeal ration resulted in 3.58 $\log_{10}$ CFU g^{-1}, which was numerically higher than the CS treatment. Chickens fed the BF ration resulted in *Clostridium* spp. counts of 4.15 $\log_{10}$ CFU g^{-1}; numerically higher than barley and fishmeal alone and significantly ($p<0.05$) higher than the CS treatment.

Growth parameters: For Trial 2, individual body weight gain (BWG) and feed conversion (F:G) were determined and results are displayed in Table 4. Birds fed the CS 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 BF. Chickens sampled from the BF 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 CS and barley and significantly ($p<0.05$) lower than the BF combination. Chickens fed BF had the most efficient feed conversion, 1.06, significantly ($p<0.05$) lower than the barley and CS diets. Corn-SBM (1.11) had the poorest feed conversion, which was significantly ($p<0.05$) different from birds fed fishmeal or BF. 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 for trial 2 at day 0 are shown on Table 8. The rapid kit identified (No. of samples): *Clostridium perfringens, C. sporogenes*, *C. innocuum*, *C. hastiforme* and *C. tertium*

Table 5: Trial 1: RapID-ANA II confirmation results for environmental samples, feed samples and chick samples processed using the fung double tube, 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)
Feed-expanded	2	*C. innocuum*, *C. perfringens* (2)
Feed-CS	3	*C. perfringens*
Feed-SAA	2	*C. perfringens*
Feed-BF	4	
Chicks	3	*C. innocuum* (3), *L. acidophilus*

[1]Treatment diets; Expanded: Expanded corn, CS: Corn-SBM, SAA: Low CP/high synthetic AA, BF: Barley/fishmeal

Table 6: Trial 1: RapID-ANA II confirmation results for environmental samples and intestinal samples of broilers fed different dietary ingredients processed using the fung double tube, day 21

Treatments	Sample	Unidentified	Isolates
Expanded	Litter	2	
	Feed	1	*C. innocuum*
	Feeder	1	*C. perfringens*
	Wall	2	
	Intestines	12	*C. perfringens* (2), *L. acidophilus* (3)
	Litter		*C. innocuum*, *L. acidophilus*
CS	Feed	2	
	Feeder	1	*C. innocuum*
	Intestines	11	*Bifidobacterium*, *C. perfringens*, *C. subterminale*, *L. acidophilus* (4)
	Feed	1	*C. innocuum*
SAA	Feeder	2	*E. aerofaciens*
	Wall	1	*C. hastiforme*
	Intestines	7	*A. mayeri*, *C. butyricum*, *C. perfringens* (2), *C. subterminale*, *C. tertium* (2), *L. acidophilus* (4)
BF	Feed	1	*C. innocuum*
	Feeder	2	*C. perfringens*, *C. innocuum*
	Intestines	7	*Bifidobacterium* (3), *C. perfringens*, *L. acidophilus* (6), *L. fermentum*
	Entrance	1	*E. aerofaciens*
Floor	Midway	1	*L. acidophilus*
	End	1	*C. innocuum*

[1]Treatment diets; Expanded: Expanded corn, CS: Corn-SBM, SAA: Low CP/high synthetic AA, BF: Barley/fishmeal

from floor samples. In wall samples, *C. tetani* and *C. perfringens* (3) were found. Feeder samples resulted in *C. perfringens*, *C. butyricum* and *Lactobacillus minutus*. As far as feed samples, *C. perfringens* (4) was found in all diets, except BF. *Clostridium innocuum* was also identified in expanded corn. Broiler chick intestinal samples were identified as *C. innocuum* (3) and *Lactobacillus acidophilus*. On day 0, 28 samples did not code for identification. Confirmation results for environmental samples after 21 days are shown in Table 3. 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 CS treatment, *C. innocuum* was found in the litter and feeder. The litter sample was also positive for *L. acidophilus*. In the SAA 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 BF 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 *Eubacterium 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 Trial 1 are shown in Table 5. A total of 37 samples were not identified by the rapid kit. In the CS 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 SAA 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 BF ration were identified as *C. perfringens*, *bifidobacterium* (3), *L. acidophilus* (6) and *L. fermentum*.

In Trial 2, confirmation results for diet samples are found in Table 6. Identifications were not possible for a total of five samples. *Clostridium tetani* was found in the fishmeal diet. The BF combination was positive for *C. innocuum*, *L. casei* and *L. acidophilus*. Broiler chicken intestinal samples identification results are displayed on Table 7. Birds fed diets with CS were positive for *C. perfringens*, *C. subterminale* and

Table 7: Trial 2: RapID-ANA II confirmation results for diet samples (day 0) and intestinal samples (day 21) of broilers fed different dietary ingredients processed using the fung double tube

Treatments	Sample	Unidentified	Isolates
CS	Intestines	14	*C. perfringens*, *C. subterminale*, *E. limosum*
Barley	Intestines	19	
	Feed	2	
Fishmeal	Intestines	21	*C. clostridioforme*, *L. acidophilus*
	Feed	2	*C. tetani*
BF	Intestines	16	*C. innocuum*, *C. perfringens*
	Feed	1	*innocuum*, *L. casei*, *L. acidophilus*

[1]Treatment diets; CS: Corn-SBM, BF: Barley/fishmeal

Table 8 Trial 2 - Environmental and bird samples RapID-ANA II 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*

E. limosum. Samples of broiler chickens fed fishmeal were identified as *C. clostridioforme* and *L. acidophilus*. Lastly, the BF 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 form spores. In our first experiment, swab samples obtained from feeders, pen walls and floors were contaminated with *Clostridium* spp. at the beginning of the trial, as well as, after 21 days. These results resonate with the findings of Craven *et al.*[28]. 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 *Clostridium* spp. 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 take place in order to obtain counts of *Clostridium* spp. may have jeopardized accurate sampling. As far as air samples, a longer sampling time should be implemented to evaluate low *Clostridium* spp. counts. Samples of the CS rations fed in Trial 1 and 2 did not show colonies of *Clostridium* spp. This finding may be of interest since some researchers have contemplated that digested corn may contain certain anti-*Clostridium* substances[25]. Broiler diets formulated with barley, fishmeal and BF in Trial 2, had approximately 2.5 $\log_{10}$ CFU g^{-1} of *Clostridium* spp., resulting in a viable contamination source. The diet containing expanded corn was the only one with positive counts of *Clostridium* spp. in Trial 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 Trial 1, the diet with highest concentration of *Clostridium* spp. 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, Trial 2 was designed to better understand if barley and fishmeal alone would increase *Clostridium* spp. counts and if a cumulative effect existed. As other researchers have found, fishmeal and barley alone increased *Clostridium* spp. concentrations in broiler chicken intestines. Furthermore, our results indicate that a synergistic effect was found, since the highest concentrations of *Clostridium* spp. were found in the BF diet.

Researchers have investigated the influence of protein source and cereal grains on *C. perfringens*[19] found that birds fed proteins of animal source, such as fishmeal, had increased counts of *C. perfringens*. Similar results have been documented in previous studies[13,18]. Yet another protein source implicated in exacerbating *C. perfringens* counts is

potato concentrate[9,19]. *Clostridium perfringens* has been shown to thrive in feed ingredients high in methionine and glycine[16,19]. 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[15,20,21]. Hofshagen and Kaldhusdal[37] found higher concentrations of *C. perfringens* in diets formulated with barley instead of corn yet 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[10] 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[3,17,38-40].

As far as dietary antimicrobial effects, *Clostridium* spp. counts were similar in Trial 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.*[11], 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* presumptive colonies. In Trials 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 : colony enzyme production and database updating. Ruengwilysup *et al.*,[41] 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 *Clostridium* spp. 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, considering that the generation time for some *C. perfringens* strains is less than 8 min, the database may classify a wild-type *C. perfringens* strain as unidentifiable.

CONCLUSION

This study illustrates that the FDT may be a powerful tool in enumerating *Clostridium* spp. from chicken intestines and environmental samples. Furthermore, the FDT detected differences in *Clostridium* spp. counts from broiler chickens fed different experimental diets containing feed ingredients such as, barley and fishmeal, which have been shown to exacerbate *C. perfringens*.

SIGNIFICANCE STATEMENT

This study describes the validation and use of a laboratory method that allows monitoring the environment, feed and broilers for NE. Researchers and producers will be able determine the levels of NE associated with broilers during the production phase so that the birds may be raised in a healthy manner. These findings will useful for producing broilers with less antibiotics while improving welfare and reducing mortality.

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