Efficacy of multistrain direct-fed microbial and phytogenetic products in reducing necrotic enteritis in commercial broilers


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ABSTRACT Our laboratory is evaluating the efficacy of direct-fed microbials (DFM) and phytogenic products to control Clostridium perfringens, a gram-positive organism associated with decreased performance and morbidity and mortality associated with necrotic enteritis, as well as some recent human food safety issues. Three experiments were conducted to evaluate a DFM (PoultryStar) and a phytogenic product (PEP125), which were administered to birds from day of hatch until termination (d 25) via the drinking water or through supplementation to a wheat-corn diet, respectively. Each experiment contained a nonchallenged negative control and a positive control wherein birds were immunocompromised with a 10× dosage of infectious bursal disease vaccine at 14 d of age and subsequently gavaged with C. perfringens (10⁷ cfu/mL) daily for 3 consecutive days starting on d 17. Intestinal lesions, mortality, and log₁₀ values of C. perfringens in the probiotic and phytogenic treatment groups were found to be lower (P < 0.05) than those observed in the positive controls. These experiments suggest that the DFM and the phytogenic product could be used as potential alternatives to help control C. perfringens and necrotic enteritis.

Key words: Clostridium perfringens, chicken, direct-fed microbial, phytogenetics, necrotic enteritis

INTRODUCTION

Clostridium perfringens is a spore-forming, gram-positive, rod-shaped bacterium that grows in anaerobic conditions. These bacteria can be found in many areas of our environment and are ubiquitous in nature. Some of the primary host reservoirs of these bacteria include humans, cats, cows, pigs, sheep, and chickens (Maier et al., 2000). In animal agriculture, poultry and beef products are the predominant food commodities of the meat industry, with annual human food consumption on a per capita basis of 149.6 and 138.6 kg, respectively, in the United States (Food Consumption, 2003). Clostridium perfringens is prevalent in commercial poultry, with 75 to 95% of the gastrointestinal tract of broilers having tested positive for C. perfringens in previous studies (Tschirdevahn et al., 1991; Miwa et al., 1997; Craven et al., 2001a,b). Processed poultry meat has also been shown to have relatively high numbers of C. perfringens from 8 to 84% (Miwa et al., 1997; Craven et al., 2001b). This enteric pathogen, which is found throughout broiler production, may be transmitted to humans through the consumption of contaminated poultry products (Labbe, 1991; Craven et al., 2001a,b).

For the commercial poultry industry, controlling the levels of C. perfringens is an important issue because of the economic cost of infected flocks. It has been estimated that, worldwide, C. perfringens costs the international poultry industry in excess of $US 2 billion per year (Kaldhusdal and Løvland, 2000). Clostridium perfringens is the etiologic agent of the disease necrotic enteritis (NE). The clinical signs of this disease include depression, decreased appetite, diarrhea, and severe necrosis of the gastrointestinal tract (Calnek, 1997). Understanding the disease progression of NE has been very difficult due to its complexity and several predisposing factors such as dietary components, immunosuppression, mechanical irritation of the gut, and sudden changes in gut microflora that appear to contribute to this syndrome (Smith, 1965; Elwinger et al., 1992; Calnek, 1997).

The gastrointestinal microbial community is a sophisticated association of many different species of bacteria with the flora differing from host to host. Interestingly, individual variations in microbial flora between birds may account for some of the differences in uniformity, bird performance, as well as disease susceptibility observed in commercial flocks. Denaturing gradient gel electrophoresis data from van der Wielen et al. (2002) suggest that birds from the same flock share bacterial
population similarities of only 50%. These similarities are surprisingly small because birds grown in the same environment are, theoretically, exposed to the same environmental microbial flora. The low microbiologic similarities between birds may suggest that host factors such as host immune status (Toivanen et al., 2001), genotype, specific host receptors, or bacterial communication systems (Zoetendal et al., 2001) contribute significantly to the bacterial profile of the intestinal tract of individuals.

Probiotic direct-fed microbial (DFM) products are composed of beneficial intestinal microflora from healthy adult chickens and are administered to neonatal chickens for the prevention of intestinal colonization by pathogens (Nurmi and Rantala, 1973; Snoeyenbos et al., 1979; Weinack et al., 1979; Barnes et al., 1980; Mead and Impey, 1986; Corrier et al., 1995). Direct-fed microbials are live, naturally occurring microorganisms that contribute to the health and balance of the microbiota in the intestinal tract. Direct-fed microbials are given orally to poultry in the first few days of life and in transitional (stressful) periods to aid in the maintenance of the health and performance of the birds. Prebiotics are nondigestible products and their effect in the diet is to promote the growth and proliferation of beneficial bacteria in the intestinal tract, thus enhancing the effect of DFM bacteria. The prebiotics used in the present investigation were phytogenic (PHYTO) feed additives based on a combination of essential oils and prebiotic substances formulated to maintain a healthy gut at times of digestive stress. A healthy gut not only affects nutrient utilization by the animal but also has a considerable influence on the immune status of the animal. Poor digestion leads to increased amounts of nutrients available for pathogenic bacteria to use and rapidly multiply in the digestive tract, which can lead to enteric diseases. The present investigation was designed to evaluate the effects of a DFM product and a phytogenic product during an experimentally induced case of NE.

MATERIALS AND METHODS

Experimental Birds

Ross × Ross straight-run broiler chicks were obtained from a local commercial hatchery on clay of hatch and were placed on clean pine shaving litter. Birds were reared in 2.4 m × 1.2 m pens, allowing 0.12 m² of pen space per bird. Chicks were provided with water ad libitum and a wheat-corn-soy-based broiler starter diet at 55, 15, and 22%, respectively. The diet was further balanced following the NRC recommended levels for DL-methionine, L-lysine, animal-vegetable fat blend, limestone, monocalcium PO₄, salt, trace minerals, and vitamins (NRC, 1994). High concentrations of wheat were used in the diet because this kind of diet has been shown to exacerbate the outbreak of NE (Johnson and Pinedo, 1971; Truscott and Al-Sheikhly, 1977; Branton et al., 1987; Riddell and Kong, 1992). Technical procedures of this study were approved by the Institutional Animal Care and Use Committee.

Immunosuppression Vaccine Administration

As described previously, a commercial bursal disease vaccine was used as an immunosuppressant in the present investigations (McReynolds et al., 2004). All birds were administered the vaccine on d 14, at a level 10× the recommended dose of the manufacturer via ocular route to immunocompromise the chicks. Challenge doses, at these concentrations, were chosen based on previous research (data not shown) and birds have been known to show signs of the disease state.

C. perfringens Administration

Four field isolates of C. perfringens (type A) were collected from commercial flocks having NE, in different geographical locations (1 isolate from Texas and 2 isolates from Georgia), and were isolated and cultured separately, then combined and provided to the appropriate treatment groups (McReynolds et al., 2004). For challenge, the isolates were grown in thioglycollate medium for 12 h, and all chicks except for the negative control were challenged via oral gavage (3 mL) with 10⁵ cfu of C. perfringens/mL for birds in experiment 1 and 10⁷ cfu of C. perfringens/mL for birds in experiments 2 and 3. Birds were administered C. perfringens twice daily for 3 consecutive days beginning on d 17. Again, challenge doses, at these concentrations, were chosen based on previous research (data not shown) and have been known to show signs of NE with intestinal lesions.

Experimental Design

In experiment 1, birds were randomly assigned to one of the following groups: negative control; positive control; PHYTO blend 1, 2, 3; or a DFM blend 1, 2 (n = 50 birds for all groups). Birds were fed the control diet or a diet that contained one of the PHYTO blends 1, 2, or 3. These products contain a proprietary PHYTO mixture containing, but not limited to, essential oil (citrus, oregano, annise) plant extracts and fructooligosaccharides (PEP125, Biomin, San Antonio, TX). The 3 PHYTO products contained all of the active ingredients with varying concentrations of each. The products were administered in the feed at a concentration of 1 kg/ton (US) of feed from day of hatch until termination (d 25) of the experiment. The DFM blend 1 (PoultryStar, Biomin) contained 1.3 × 10¹¹, and DFM blend 2 contained 1.4 × 10¹² of lactic acid bacteria including Enterococcus faecium, Pediococcus acidilactici, Bifidobacterium animalis, and Lactobacillus reuteri. The products were administered through the drinking water at a concentration of 20 g/1,000 birds per day, which
delivers $1 \times 10^8$ cfu/mL for DFM blend 1 and $1 \times 10^9$ for DFM blend 2 from day of hatch until termination (d 25). The calculated water consumption was based on the NRC guidelines and averaged 225, 480, and 725 mL/bird per week (NRC, 1994). In experiment 1, we evaluated several parameters throughout the study for each treatment group: total mortality ($n = 50$), microbial populations $\log_{10}$ cfu of *C. perfringens*/g ($n = 10$), and the development of clinical lesions ($n = 24$).

In 2 replicate studies, experiments 2 and 3, birds were randomly assigned to one of the following groups: negative control, positive control, probiotic blend 1, PHYTO blend 1, or the combination of both products. The experimental procedures previously mentioned in experiment 1 were followed for both experiments. In experiment 2, we evaluated several parameters including the following: total mortality ($n = 37$ positive controls, 74 for treatment groups), microbial populations $\log_{10}$ cfu of *C. perfringens*/g ($n = 10$), and the development of clinical lesions ($n = 24$). In experiment 3, the same parameters were measured: total mortality ($n = 50$ positive controls, 100 for treatment groups), microbial populations $\log_{10}$ cfu of *C. perfringens*/g ($n = 10$), and the development of clinical lesions ($n = 40$).

**Bacterial Culture**

To quantitatively measure populations of *C. perfringens*, a section of the small intestine approximately 15 cm in length, just cranial to Meckel’s diverticulum, was removed. Each sample was placed in 10 mL of anaerobic thioglycollate, stomached for 30 s, and a 0.5-mL aliquot of intestinal digesta was removed and placed into 4.5 mL of thioglycollate medium. Ten-fold serial dilutions were performed, plated on Shahidi-Ferguson perfringens agar, and incubated (24 h at 37°C). All of the *C. perfringens* culture work was performed in an anaerobic hood. Plates containing colonies exhibiting typical morphology with more than 30 or less than 300 colonies were counted and recorded.

**NE Lesion Scores**

To evaluate gross lesions associated with NE, the jejunum and ileum approximately 10 cm cranial and dorsal to Meckel’s diverticulum were examined. Lesion scores were recorded using the following criteria (Prescott et al., 1978): 0 = no gross lesions, normal intestinal appearance; 1 = thin-walled or friable, gray appearance; 2 = thin-walled, focal necrosis, gray appearance, small amounts of gas production; 3 = thin walled, sizeable patches of necrosis, gas-filled intestine, small flecks of blood; 4 = severe extensive necrosis, marked hemorrhage, large amounts of gas in intestine.

**Statistical Analysis**

Mortality among all treatment groups was compared using the $\chi^2$ test of independence ($P \leq 0.05$). All mortality data were based on the total number of birds per treatment group for individual experiments; all the other measured parameters were subsets of treatment groups, for individual experiments. Bacterial counts ($\log_{10}$ units) were analyzed with the PROC MIXED procedure in SAS and adjusted for multiple comparisons using the Tukey option. To evaluate the lesion scores in the present investigation, the row mean scores were compared using the Cochran-Mantel-Haenszel testing PROC FREQ. The Cochran-Mantel-Haenszel test showed significant differences ($P \leq 0.05$), and the data were further analyzed using a nonparametric ANOVA (Kruskal-Wallis) by ranking the scores, applying the mean to ties, and running a PROC GLM on the ranks, allowing the treatment groups to be compared by the mean ranks (SAS Institute, 1996).

**RESULTS AND DISCUSSION**

Many different biological factors, including pathogens, can cause disease. Many different types of microorganisms, such as parasites, viruses, and bacteria, can be pathogenic. Several pathogens that cause serious illness are in the genus *Clostridium*, including *Clostridium botulinum*, *Clostridium chauvoei*, *Clostridium tetani*, *Clostridium septicum*, and *C. perfringens*. Many of these organisms are opportunistic pathogens, and given the appropriate conditions, these bacteria will grow and flourish. A commensal intestinal microflora protects the host from these pathogens. However, if the commensal flora of the gut is disturbed, these pathogens can grow and cause disease such as NE (Van Immerseel et al., 2004).

In experiment 1, we evaluated several different PHYTO blends and probiotic products to determine the optimal blend for efficacy in reducing NE intestinal lesion development. The data from experiment 1 showed that PHYTO blends 1 and 3 and DFM blends 1 and 2 were the most efficacious ($P < 0.05$) in reducing the severity of lesion scores when compared with the positive control, with mean lesion scores of 0.58, 0.75, and 0.29, 0.83, respectively, compared with 1.33 in the positive control (Table 1). It is important to note the shift in lesion scores when evaluating the percentage of clinical intestinal lesion scores of the treatment groups compared with the positive controls. In experiment 1, more birds had a lesion score of 0 in the PHYTO blend 1 (63%) and DFM blend 2 (75%) treatment groups when compared with the positive controls (13%). This downshift in lesion score severity is important because of intestinal integrity. If intestinal lesion scores can be reduced, the infected birds have a much greater chance of recovering from the disease. Although these birds will weigh significantly less when they recover from the disease, they will be marketable. Both of these products aided in the reduction of severity of lesions and promoted the maintenance of intestinal integrity. Mortality was also reduced ($P < 0.05$) in both the PHYTO blend 1 and DFM blend 1 treatment groups when compared
Table 1. An evaluation of direct-fed microbials (DFM) or phytogenic (PHYTO) blend administration in birds experimentally infected with \textit{Clostridium perfringens} and the development of clinical lesions associated with necrotic enteritis, experiment 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Lesion score $^3$</th>
<th>Mortality $^3$</th>
<th>Log$_{10}$ cfu/g $^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>75</td>
<td>21</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0.29$^c$</td>
<td>0/50* (0%)</td>
<td>1.00$^c$</td>
</tr>
<tr>
<td>Positive control</td>
<td>13</td>
<td>50</td>
<td>29</td>
<td>8</td>
<td>0</td>
<td>1.33$^a$</td>
<td>13/50 (26%)</td>
<td>3.42$^a$</td>
</tr>
<tr>
<td>PHYTO blend 1</td>
<td>33</td>
<td>46</td>
<td>13</td>
<td>4</td>
<td>4</td>
<td>0.58$^b$</td>
<td>4/50* (8%)</td>
<td>2.16$^b$</td>
</tr>
<tr>
<td>PHYTO blend 2</td>
<td>35</td>
<td>50</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>1.00$^b$</td>
<td>7/50 (14%)</td>
<td>2.79$^b$</td>
</tr>
<tr>
<td>PHYTO blend 3</td>
<td>36</td>
<td>50</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0.75$^b$</td>
<td>3/50* (6%)</td>
<td>1.90$^b$</td>
</tr>
<tr>
<td>DFM blend 1</td>
<td>36</td>
<td>50</td>
<td>8</td>
<td>0</td>
<td>4</td>
<td>0.20$^c$</td>
<td>8/50 (16%)</td>
<td>2.91$^c$</td>
</tr>
<tr>
<td>DFM blend 2</td>
<td>75</td>
<td>21</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0.83$^b$</td>
<td>6/50 (12%)</td>
<td>1.90$^b$</td>
</tr>
</tbody>
</table>

$^a$-'c' Means within the same column with no common superscripts differ significantly ($P \leq 0.05$).

$^1$Treatment groups represented by the PHYTO blends 1, 2, and 3 administered in the feed and the DFM blends 1 and 2 administered in the water or from d 1.

$^2$Lesion score is represented by the mean of treatment subset (n = 24) with the MS error.

$^3$Mortality is represented by incidence data compared with the positive control ($* P \leq 0.05$, n = 50).

$^4$Log$_{10}$ cfu/g is represented by the mean of treatment subset (n = 10).

with the positive control, with 8 and 6%, respectively, compared with 26% mortality in the positive control treatment. The log$_{10}$ \textit{C. perfringens}/g of intestinal contents was significantly reduced in the DFM blend 1 group with a log$_{10}$ value of 1.90 compared with 3.42 in the positive control.

In experiment 2 and 3, we refined the number of phytogenic and DFM groups to PHYTO blend 1 and DFM blend 1 to replicate the findings from experiment 1. In both replicate experiments, we observed similar findings in that lesion scores were reduced as well as mortality and log$_{10}$ values of \textit{C. perfringens}, even under higher \textit{C. perfringens} challenge levels (10$^7$ vs. 10$^8$ for experiment 1). In experiment 2, a numerical reduction in the mean lesion score in the PHYTO (0.96) and DFM (0.98) treatment groups and a significant reduction in the combined treatment group (0.78) was observed compared with the positive control (1.28; Table 2). Mean lesion scores in the PHYTO, DFM, and combination groups in experiment 3 were also significantly reduced ($P < 0.05$) when birds were supplemented with the PHYTO, DFM, and combination groups with scores of 1.2, 1.2, and 1.3, respectively, when compared with the positive control score of 2.15 (Table 3). Mortality, in both experiments 2 and 3, was numerically reduced from 6 to 10%, depending on treatment group. Again, there were reductions in log$_{10}$ values ($P < 0.05$) in all of the treatment groups in experiment 2, with mean average log$_{10}$ values of \textit{C. perfringens} 3.69, 3.44, and 3.13 in the PHYTO, DFM, and combination groups, respectively, when compared with the positive control mean value of 4.79. In experiment 3, the PHYTO treatment group had a reduction ($P < 0.05$) in log$_{10}$ values with a mean of 2.87. Interestingly, population levels of \textit{C. perfringens} are unaltered in some of the experimental groups; however, intestinal lesion scores and mortality are reduced.

The probiotic strains \textit{Enterococcus sp.}, \textit{Pediococcus sp.}, \textit{Bifidobacterium sp.}, and \textit{Lactobacillus sp.} support the establishment and stabilization of a beneficial and protective gut microflora and are able to selectively exclude pathogens from colonization due to the fast proliferation, colonization, and acidification in the gut. The beneficial bacteria in the gastrointestinal tract play several key roles in animal health. There are several advantages in the development of a normal microflora: these bacteria aid in colonization resistance, competition for intestinal attachment sites, and stimulation

Table 2. An evaluation of direct-fed microbials (DFM) or phytogenic (PHYTO) blend administration in birds experimentally infected with \textit{Clostridium perfringens} and the development of clinical lesions associated with necrotic enteritis, experiment 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Lesion score $^3$</th>
<th>Mortality $^3$</th>
<th>Log$_{10}$ cfu/g $^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>76</td>
<td>16</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0.32$^c$</td>
<td>3/37* (8%)</td>
<td>1.57$^c$</td>
</tr>
<tr>
<td>Positive control</td>
<td>32</td>
<td>20</td>
<td>36</td>
<td>12</td>
<td>0</td>
<td>1.28$^a$</td>
<td>10/37 (27%)</td>
<td>4.79$^a$</td>
</tr>
<tr>
<td>DFM</td>
<td>42</td>
<td>28</td>
<td>22</td>
<td>6</td>
<td>2</td>
<td>0.88$^b$</td>
<td>13/74 (17%)</td>
<td>3.41$^b$</td>
</tr>
<tr>
<td>PHYTO</td>
<td>44</td>
<td>26</td>
<td>20</td>
<td>10</td>
<td>0</td>
<td>0.96$^c$</td>
<td>15/74 (20%)</td>
<td>3.69$^c$</td>
</tr>
<tr>
<td>Combination</td>
<td>50</td>
<td>32</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td>0.78$^b$</td>
<td>12/74 (16%)</td>
<td>3.13$^b$</td>
</tr>
</tbody>
</table>

$^a$-'c' Means within the same column with no common superscripts differ significantly ($P \leq 0.05$).

$^1$Treatment groups represented by the PHYTO blends 1, 2, and 3 administered in the feed and the DFM blends 1 and 2 administered in the water or from d 1.

$^2$Lesion score is represented by the mean of treatment subset (n = 24) with the MS error.

$^3$Differences in mortality are represented by incidence data compared with the positive control ($* P \leq 0.05$, n = 37 for controls and 74 for treatments).

$^4$Log$_{10}$ cfu/g is represented by the mean of treatment subset (n = 10).
that the pathogenic bacteria are still in abundance and cell (Titball, 1993; Murray et al., 1999). When evaluating the log, cfu values of C. perfringens, we clearly see that the pathogenic bacteria are still in abundance and maintained at high concentrations in the gastrointestinal tract in all groups. However, even in relatively high numbers, these bacteria and their toxins are having less of an effect on the intestinal cells, as can be seen in the reduced lesion scores associated within the phytogenic and probiotic treatment groups. C. perfringens is ubiquitous in nature and a resident of the normal intestinal ecology. Understanding the trigger mechanisms that cause this bacteria to grow rapidly and produce copious amounts of toxins that cause disease needs further investigation. Understanding this complex intestinal ecology and how to promote homeostasis is difficult because of the multitude of complex interactions between host and pathogen as well as commensal microbes.

Necrotic enteritis in poultry has been shown to be controlled by growth-promoting antibiotics such as bacitracin methylene disalicylate and narasin. In a recent study, the combination of these antibiotics markedly reduced the clinical signs associated with NE (Brennan et al., 2001). Necrotic enteritis has been controlled using antimicrobials for many years in the poultry industry. However, if antibiotic compounds are removed from the market, this disease could prove to be costly to the poultry industry. A myriad of physiological parameters must be evaluated to better understand the interactions of phytogenic and DFM products on the development of NE. These nonantibiotic compounds have been shown to significantly increase many positive attributes in the poultry industry from increasing production parameters to the prevention of disease caused by pathogenic bacteria. Furthermore, they have been shown to significantly reduce the effects of C. perfringens in the present investigations. The data suggest that these products may provide the poultry industry with an alternative management tool that has the potential to promote better intestinal health and decrease monetary losses due to C. perfringens.

REFERENCES
