Dietary Encapsulated Glycine Influences *Clostridium perfringens* and Lactobacilli Growth in the Gastrointestinal Tract of Broiler Chickens\(^1\)–\(^3\)

J. P. Dahiya,\(^4\) Dirk Hoehler,\(^5\) Andrew G. Van Kessel,\(^4\) and Murray D. Drew\(^4\)*

\(^4\)Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5A8 and \(^5\)Degussa Corporation, Kennesaw, GA 30144

Abstract

Three experiments were conducted to determine whether there is a causative relation between dietary glycine concentration and intestinal *Clostridium perfringens* growth in broiler chickens. Expt. 1 showed that glycine concentrations were higher (\(P < 0.05\)) in jejunum and ileum of birds fed fat-encapsulated glycine compared with crystalline glycine. In Expt. 2, 2 cages of 6 birds were assigned to 1 of 6 experimental diets formulated to contain 7.6 and 10.6, 17.8 and 40.6, 27.8 and 30.6, 37.8 and 20.6, 47.7 and 10.6, and 7.8 and 50.6 g/kg total glycine and proline, respectively, provided primarily by supplementation with encapsulated glycine or proline as required. In Expt. 3, 12 groups of 6 birds were fed 4 different diets supplemented with encapsulated glycine to achieve 7.6, 21.0, 34.3, or 47.7 g/kg total glycine. The birds were orally challenged with *C. perfringens* type A on d 1 and d 14–21 and killed on d 28. In Expt. 2, *C. perfringens* populations were higher (\(P < 0.05\)) in ileum and cecum of birds, which received either 37.8 or 47.7 g/kg total glycine compared with those fed 7.6 g/kg glycine. In Expt. 3, *C. perfringens* numbers were higher (\(P < 0.05\)) in ileum of birds fed either 34.3 or 47.7 g/kg dietary glycine than those given either 7.6 or 21.0 g/kg glycine. Conversely, lactobacilli counts in ileum and cecum were significantly lower in birds fed the higher levels of glycine in both experiments. High *C. perfringens* colonization and high intestinal lesion scores were associated with reduced performance (\(P < 0.05\)). We conclude that glycine is an important determinant of *C. perfringens* growth in the intestinal tract of broiler chickens. J. Nutr. 137: 1408–1414, 2007.

Introduction

Enteric pathogens result in huge losses to the poultry industry annually, and their control or reduction could potentially save the producers millions of dollars. Recognized as the causative agent of necrotic enteritis (NE),\(^6\) *Clostridium perfringens* is 1 such pathogen. *C. perfringens* is a nearly ubiquitous Gram-positive, spore-forming, extremely prolific, toxigenic anaerobic bacteria affecting many warm-blooded animals, including humans (1). High populations of *C. perfringens* may be present in the intestinal tract of animals with no visible signs of disease, thus contradicting Koch’s postulate that a disease-causing organism should not be present in healthy individuals. Most workers, therefore, consider various predisposing factors to be of major importance in spontaneous outbreaks of NE in poultry (2). Despite considerable investigation of this disease, the predisposing factors that promote overgrowth of *C. perfringens*, excessive release of \(\alpha\) toxin and thus subsequent progression to disease, are numerous and ill defined. The long list of contributing factors includes management and environmental conditions (3–5), stress and immunosuppression (6,7), coinfection with *Eimeria* spp. (8,9), and diet composition. The estimated cost of NE to the poultry industry is as much as US$ 0.05 per bird, with total global loss estimated at almost US$ 2 billion (10,11). Others have suggested this is an underestimate, given the difficulty in diagnosing mild forms of NE (12). Concerns also arise over the high contamination rates of poultry by *C. perfringens* and risk of transmission to the food chain, posing public health problems (13). These factors have stimulated interest in finding alternative management or dietary strategies to control the incidence and severity of NE in postantibiotic era.

The physical and chemical attributes of diet can modify the gastrointestinal microbial ecology of birds and are an important determinants of NE (5,14,15). Birds fed diets based on wheat,
rye, oats, or barley have increased risk of NE compared with birds fed maize-based diets (14, 16, 17). Proteins of animal origin are favorable substrates for clostridial growth and high concentrations in broiler feeds are often associated with NE (5, 18–20). Although there is sufficient evidence regarding the mechanism(s) behind the effect of cereal grains on C. perfringens growth and NE, relatively little information is available in peer-reviewed literature regarding the factors or mechanisms responsible for the increased incidence of NE in broilers fed high-protein diets.

Previous work has shown that there is a significant correlation between certain amino acids, especially glycine, and C. perfringens numbers and/or α toxin production (19–23). Drew et al. (19) reported a positive association between crude protein (CP) derived from fish meal and numbers of ileal and cecal C. perfringens, but no such association existed for soy-derived proteins. Glycine and methionine levels are higher in fish meal than in soy concentrate and these amino acids are known to stimulate C. perfringens growth and phospholipase C production in vitro (22, 24–26). A significant positive correlation of dietary glycine with C. perfringens numbers in ileum and cecum of broiler chickens when fed different plant and animal protein-based diets has been documented (20). Recently, we reported a positive correlation between dietary protein-bound glycine content and gut C. perfringens growth in broiler chickens when gelatin was used as protein source; however, because gelatin also contains high levels of proline, a causative link between glycine and clostridial growth could not be conclusively established (21). This study was therefore conducted to determine whether a causative relation exists between dietary glycine/proline and intestinal clostridial growth in broiler chickens. Because crystalline glycine/proline is rapidly absorbed in the duodenum, it is largely unavailable to C. perfringens populations in the distal gut. Fat (hydrogenated palm oil)-encapsulated glycine/proline was therefore used to slowly release the amino acid along the entire length of the gut. The purpose of this study was to examine the effect of feeding encapsulated glycine- and proline-based diets on intestinal C. perfringens colonization and NE lesion scores in broiler chickens.

Materials and Methods

Experimental protocols were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with recommendations of the Canadian Council on Animal Care as specified in the Guide to the Care and Use of Experimental Animals (27).

Experimental birds, diets, and design

**Expt. 1.** This experiment was conducted to determine the effect of fat encapsulation on the release of glycine in the intestinal tract of broiler chickens. A total of 36 1-d-old male Ross broiler chicks (Gallus domesticus) were obtained (Lilydale Hatchery), housed randomly in 4 electrically heated battery cages and consumed a medicated, ideal based starter crumble used in Expt. 1 (Supplemental Table 1). On d 1, birds were weighed and randomly reassigned to 1 of the 12 battery cages at 6 birds per cage. Two cages were assigned in a complete randomized block design to 1 of the 6 ideal protein-balanced (28) experimental diets (Supplemental Table 3) until the end of experiment (d 28). Diets were formulated to contain 7.6 and 10.6 (control2), 17.8 and 40.6 (G1P3), 27.8 and 30.6 (G2P2), 37.8 and 20.6 (G3P1), 47.7 and 10.6 (G4P0), and 7.8 and 50.6 (G5P4) g/kg total glycine and proline, respectively, provided primarily by supplementing with encapsulated glycine or proline as required (JEFO Nutrition). The diets were isoenergetic (12.8 MJ/kg ME) and contained 11.6 g/kg lysine with other essential amino acid levels formulated to be within 10% of the ideal protein ratio. Canola oil was used to make the diets isocaloric, because encapsulated material contains 625.0 g/kg each extract. The diets met or exceeded the NRC nutrient requirements for broiler chickens for all other nutrients (29).

**Expt. 2.** A total of 72 1-d-old conventional male broiler chicks (G. domesticus, Ross 308) were obtained (Lilydale Hatchery) and placed randomly into 4 electrically heated battery cages (18 birds per cage). On d 1 through 14 of the experiment, birds were provided with the same corn-based starter crumble used in Expt. 1 (Supplemental Table 1). On d 14, birds were weighed and randomly reassigned to 1 of the 12 battery cages at 6 birds per cage. Two cages were assigned in a complete randomized block design to 1 of the 6 ideal protein-balanced (28) experimental diets (Supplemental Table 3) until the end of experiment (d 28). Diets were formulated to contain 7.6 and 10.6 (control), 17.8 and 40.6 (G1P3), 27.8 and 30.6 (G2P2), 37.8 and 20.6 (G3P1), 47.7 and 10.6 (G4P0), and 7.8 and 50.6 (G5P4) g/kg total glycine and proline, respectively, provided primarily by supplementing with encapsulated glycine or proline as required (JEFO Nutrition). The diets were isoenergetic (12.8 MJ/kg ME) and contained 11.6 g/kg lysine with other essential amino acid levels formulated to be within 10% of the ideal protein ratio. The diets met or exceeded the NRC nutrient requirements for broiler chickens for all other nutrients (29).

We followed standard management procedures for all 3 experiments during this study. Briefly, the research facility was thoroughly cleaned and disinfected prior to bird placement. The battery cages were arranged in 4 levels with a wire floor and were equipped with external feed and water troughs. The cages were continuously illuminated (24 h/d) and located in a room with controlled temperature and humidity. Room temperature was maintained according to industry standards. None of the experimental diets contained antibiotics or coccidiostats and were not pelleted. Throughout each experimental period, birds consumed feed and water ad libitum. Feed consumption and body weight for each cage was recorded for the periods d 14–21 and 21–28 in each experiment for calculation of mortality-corrected feed conversion. Degussa Canada performed amino acid analysis of the different diets.

**C. perfringens challenge**

The C. perfringens challenge model was based on that developed originally by Dahiya et al. (21). Briefly, an avian C. perfringens field strain isolated from a clinical case of NE was obtained from Dr. Manuel Chirino, College of Veterinary Medicine, University of Saskatchewan, and characterized by PCR technique as type A toxin producer. The organism was cultured anaerobically on BBL Blood Agar Base (Becton, Dickinson) containing 5.0% sheep blood and 100 mg/L neomycin sulfate (The Upjohn Company) for 18 h at 37°C, then aseptically inoculated into cooked meat medium (Difco Labs) and incubated anaerobically for 8 h at 37°C. All birds were orally challenged in the crop with 0.5 mL on d 1 and 1.0 mL on d 14–21, inclusive with this actively growing culture of C. perfringens, using a 12.0-mL syringe equipped with vinyl tubing (i.d. 0.97 mm, o.d. 1.27 mm). Bacterial counts were performed on the culture.
daily prior to inoculation and the numbers ranged from $6.38 \times 10^6$ to $7.29 \times 10^7$ colony forming units (CFU)/L.

**Pathological variables**

Birds were observed on a pen basis at least once daily for any signs of NE, e.g., huddling, diarrhea, depression, or mortality, and all birds that died during the course of experiments were necropsied to determine the cause of death. On d 28, the surviving chickens were killed by cervical dislocation, weighed, and necropsied. Intestinal tracts were removed immediately and intestinal lesions were scored without knowledge of treatment on a scale of 0–4 as described by Dahiya et al. (21). Following postmortem examination, a 1.5- to 2.0-cm-long intestinal tissue piece from the ileum with the gross NE lesion was collected in phosphate-buffered formaldehyde solution and processed routinely for paraffin embedding, sectioned at $\sim 5 \mu m$, and stained with hematoxylin and eosin.

**Enumeration of C. perfringens and lactobacilli**

In Expt. 2 and 3, the fresh intestinal contents from ileum (Meckel’s diverticulum to cm proximal to ileocecal junction) were collected aseptically into sterilized plastic dram vials and mixed well. Using sterile spatula, the subsamples were transferred into preweighed 15-mL sterile plastic tubes containing 1 mL 0.1% sterile peptone buffer with 5 g/L cysteine hydrochloride. The ceca samples were directly collected into the preweighed 15-mL sterile tubes. Both sets of samples were immediately placed and kept on ice until plated within 3 h of collection. The samples were weighed and diluted in peptone water to an initial $10^{-1}$ dilution. The 10-fold dilutions were spread in duplicate using an automated spiral plater (Autoplate, Spiral Biotech) on BBE Blood Agar Base (VWR International) containing 50 mL/L sheep blood and 100 mg/L neomycin sulfate (Upjohn) for the enumeration of C. perfringens and MRS agar (Becton, Dickinson) for the enumeration of lactobacilli. The blood agar/neomycin plates were incubated anaerobically for 24 h at 37°C and MRS agar plates were incubated anaerobically for 48 h at 37°C. The α- and β-hemolytic colonies on blood agar/neomycin plates were counted as C. perfringens with presumptive colonies being randomly picked, Gram stained, plated on Mannitol Yolk Polymixin agar (Oxoid), incubated anaerobically overnight, and examined microscopically to confirm them as C. perfringens. Bacterial counts were expressed as the log$_{10}$ CFU/g of intestinal contents.

**Samples for amino acid digestibility determination**

In Expt. 2 and 3, the remainder of the ileal contents of 3 birds from each pen was pooled, lyophilized, and analyzed for protein-bound as well as free amino acid concentrations using an oxidation and hydrolysis method (Degussa Canada).

**Statistical analyses**

In Expt. 1, the effects of 2 diets (crystalline vs. encapsulated glycine based) on the concentration of glycine in intestinal contents of birds were compared using 1-way ANOVA and differences between means were considered significant when $P < 0.05$. In Expt. 2 and 3, each cage was considered an experimental unit. Bacterial counts, growth performance, and lesion scores were analyzed using the general linear model procedure of SPSS (v.12.0). Treatment means were compared using the Ryan-Einot-Gabriel-Welsch multiple F test and were considered significantly different when $P < 0.05$.

**Results**

**Effect of encapsulation on amino acid concentration in intestinal contents.** The analyzed amino acid concentrations of the diets used in Expt. 1 were similar, including those of glycine, whether provided in crystalline or encapsulated form (Supplemental Table 2). Glycine concentration was higher ($P < 0.05$) in jejunum and ileum of birds fed encapsulated glycine compared with crystalline glycine, whereas the cecum glycine concentration did not differ between groups ($P = 0.13$) (Fig. 1). The remaining amino acids were in similar concentrations in the 2 groups at all locations in the intestinal tract (data not shown).

For diets used in Expt. 2 and 3, analyzed dietary glycine and proline concentrations were consistent with supplemented levels and the analyzed concentrations of all other amino acids were similar (Supplemental Tables 3 and 4). In agreement with the results of Expt. 1, the glycine and proline concentrations in ileal digesta collected during the challenge studies (i.e. Expt. 2 and 3) varied directly with the amount of encapsulated amino acid supplemented in the diet (Supplemental Table 5). In contrast, ileal concentrations of all other amino acids were not affected.

**Performance data.** The experimental diets were readily accepted in both challenge experiments and feed consumption did not differ between the groups during d 14–21 or d 21–28 of age. In Expt. 2 and 3, the diets did not affect weight gain and feed efficiency (FE) at the end of the d 14–21 period, whereas they did affect weight gain and FE at the end of the d 21–28 period. In Expt. 2, weight gain generally was lower in birds fed diets supplemented with encapsulated amino acids compared with the unsupplemented control2 diet and the difference was significant ($P < 0.05$) in birds receiving G3P1 and G4P0 diets (Table 1). Conversely, FE was significantly increased by encapsulated amino acid supplementation. In Expt. 3, weight gain and FE were lower ($P < 0.05$) in birds provided with UHG diets than in all other groups.

**Quantification of lactobacilli and C. perfringens.** The number of lactobacilli and C. perfringens increased from the proximal to the distal part of the intestine with the highest counts measured in the ceca. In both challenge experiments, the number of lactobacilli decreased and that of C. perfringens increased with increasing dietary glycine.

C. perfringens numbers were greater ($P < 0.05$) in the ileum and cecum of d 28-old chickens fed either G3P1 or G4P0 diets compared with those given either control2 or G0P4 diets in Expt. 2 (Table 2). Additionally, C. perfringens numbers did not differ between birds fed the lowest glycine diets with either low (control2) or high (G0P4) proline concentrations, indicating that C. perfringens growth was not influenced by dietary proline concentration. In Expt. 3, C. perfringens counts in the ileum increased from 3.12 to $4.52 \log_{10}$ CFU/g of intestinal contents as dietary glycine concentration increased from 7.6 to 47.7 g/kg and...
TABLE 1  Effect of dietary glycine and/or proline concentrations on feed intake, body weight gain, and FE of broiler chickens during d 14–21 and 21–28 of Expt. 2 and 3

<table>
<thead>
<tr>
<th>Experimental diets</th>
<th>Feed intake (g/d)</th>
<th>Weight gain (g/g feed)</th>
<th>FE</th>
<th>Feed intake (g/d)</th>
<th>Weight gain (g/g feed)</th>
<th>FE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expt. 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control2</td>
<td>76.40</td>
<td>40.07</td>
<td>0.52</td>
<td>82.75</td>
<td>52.16</td>
<td>0.63</td>
</tr>
<tr>
<td>G1P3</td>
<td>66.85</td>
<td>35.76</td>
<td>0.53</td>
<td>89.97</td>
<td>40.79</td>
<td>0.45</td>
</tr>
<tr>
<td>G2P2</td>
<td>68.09</td>
<td>36.22</td>
<td>0.53</td>
<td>94.02</td>
<td>44.42</td>
<td>0.47</td>
</tr>
<tr>
<td>G3P1</td>
<td>68.23</td>
<td>33.28</td>
<td>0.49</td>
<td>91.29</td>
<td>39.52</td>
<td>0.43</td>
</tr>
<tr>
<td>G4P0</td>
<td>70.37</td>
<td>29.49</td>
<td>0.42</td>
<td>86.61</td>
<td>40.40</td>
<td>0.47</td>
</tr>
<tr>
<td>G5P4</td>
<td>67.87</td>
<td>35.82</td>
<td>0.53</td>
<td>86.79</td>
<td>41.16</td>
<td>0.47</td>
</tr>
<tr>
<td>Pooled SEM</td>
<td>1.15</td>
<td>1.08</td>
<td>0.06</td>
<td>1.80</td>
<td>1.48</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>P pool value</strong></td>
<td>0.11</td>
<td>0.54</td>
<td>0.72</td>
<td>0.63</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Expt. 3</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control3</td>
<td>77.72</td>
<td>43.04</td>
<td>0.55</td>
<td>92.43</td>
<td>55.24</td>
<td>0.60</td>
</tr>
<tr>
<td>MG</td>
<td>72.00</td>
<td>38.95</td>
<td>0.54</td>
<td>87.72</td>
<td>49.35</td>
<td>0.58</td>
</tr>
<tr>
<td>HG</td>
<td>75.82</td>
<td>39.95</td>
<td>0.53</td>
<td>96.48</td>
<td>50.86</td>
<td>0.53</td>
</tr>
<tr>
<td>UHG</td>
<td>68.42</td>
<td>33.50</td>
<td>0.49</td>
<td>90.43</td>
<td>42.64</td>
<td>0.47</td>
</tr>
<tr>
<td>Pooled SEM</td>
<td>1.64</td>
<td>1.26</td>
<td>0.03</td>
<td>2.16</td>
<td>1.94</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>P pooled value</strong></td>
<td>0.19</td>
<td>0.21</td>
<td>0.77</td>
<td>0.77</td>
<td>0.03</td>
<td>0.01</td>
</tr>
</tbody>
</table>

1 Values are means with pooled SEM, n = 12. Within an experiment, means in a column with superscripts without a common letter differ, P < 0.05.

the birds that were fed either control3 or MG diets differed from those fed either HG or UHG diets (P < 0.05). The highest C. perfringens counts in ceca were found in birds that were fed the UHG diets. UHG, HG, and MG diets all had significantly more C. perfringens in ceca than those fed control3 diets.

In Expt. 2, the lactobacilli counts were lower (P < 0.05) in the ileum of chickens fed the G4P0 diet compared with the rest of the dietary treatments, whereas in the cecum, lactobacilli growth was significantly lower in birds fed the G1P3 diet than in those fed either control2 or G0P4 diets. However, the lactobacilli numbers did not differ in either ileum or cecum of birds receiving either control2 or G0P4 diets. In Expt. 3, the lactobacilli numbers were higher (P < 0.05) in ileum and cecum of chickens that were fed control3 diets than those fed other levels of dietary glycine (Table 2). Lactobacilli growth in ileum and cecum did not differ in birds fed MG, HG, or UHG diets.

**Clinical signs and NE lesions.** In Expt. 2 and 3, some of the birds initially became dull, depressed, and had abnormally wet droppings after we started challenging them with C. perfringens on d 14. During the course of this study, 3 birds died in Expt. 2 (from G3P1 and 1 from G1P3 groups) and 4 birds died in Expt. 3 (3 from UHG and 1 from MG groups) of causes unrelated to C. perfringens challenge. Most dead birds were in good body condition and did not have any detectable gross lesions of NE either in intestine or any other organ, with the exception of occasional petechial hemorrhages in distal jejunum and proximal ileum. Surviving birds had no apparent signs of morbidity 7–10 d postchallenge.

Some birds had very thin intestinal walls with congested mucosa and mesenteric vessels engorged with blood and had focal hemorrhagic lesions in various intestinal regions. In Expt. 3, intestines of at least 2 birds were grossly hemorrhagic throughout with blood-stained fluid in the lumen. However, typical field type lesions specific to NE were not observed in any of the birds in either experiments. In Expt. 2, intestinal lesion scores did not differ in chickens in the various groups (Fig. 2). In Expt. 3, intestinal lesion scores were higher (P < 0.05) in birds that received the UHG diet than those fed either control3 or MG diets, whereas scores were not affected by the increase in dietary glycine from 34.3 to 47.7 g/kg.

**Histological examination.** Histological examination of formalin-fixed intestinal tissues from 28-d-old broiler chickens killed in both experiments revealed no frank lesions of NE except slight edema and diffuse hemorrhages in lamina propria in some sections. There

TABLE 2  Effect of dietary glycine and/or proline concentrations on mean lactobacilli and C. perfringens populations in ileum and cecum of broiler chickens on d 28 of Expt. 2 and 3

<table>
<thead>
<tr>
<th>Experimental diets</th>
<th>Lactobacilli (log10 CFU/g intestinal contents)</th>
<th>C. perfringens (percent recovered)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ileum</td>
<td>Cecum</td>
</tr>
<tr>
<td><strong>Expt. 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control2</td>
<td>7.55a</td>
<td>8.88a</td>
</tr>
<tr>
<td>G1P3</td>
<td>7.48a</td>
<td>7.53b</td>
</tr>
<tr>
<td>G2P2</td>
<td>7.46c</td>
<td>7.95b</td>
</tr>
<tr>
<td>G3P1</td>
<td>7.45c</td>
<td>8.16b</td>
</tr>
<tr>
<td>G4P0</td>
<td>6.76b</td>
<td>7.79b</td>
</tr>
<tr>
<td>G5P4</td>
<td>7.48c</td>
<td>8.67c</td>
</tr>
<tr>
<td>Pooled SEM</td>
<td>0.058</td>
<td>0.103</td>
</tr>
<tr>
<td><strong>P pool value</strong></td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Expt. 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control3</td>
<td>7.81b</td>
<td>8.45c</td>
</tr>
<tr>
<td>MG</td>
<td>6.73c</td>
<td>7.43c</td>
</tr>
<tr>
<td>HG</td>
<td>7.06c</td>
<td>7.12b</td>
</tr>
<tr>
<td>UHG</td>
<td>6.70b</td>
<td>7.46c</td>
</tr>
<tr>
<td>Pooled SEM</td>
<td>0.132</td>
<td>0.148</td>
</tr>
<tr>
<td><strong>P pooled value</strong></td>
<td>0.05</td>
<td>0.03</td>
</tr>
</tbody>
</table>

1 Values are means with pooled SEM, n = 12. Within an experiment, means in a column with superscripts without a common letter differ, P < 0.05.

**FIGURE 2** NE lesion scores in 28-d-old broiler chickens given experimental diets (d 14–28) containing different levels of encapsulated glycine and proline. Bars represents mean ± SEM, n = 12. In Expt. 3, means without a common letter differ, P < 0.05. Lesions were scored on a 0–4 scale where 0 was apparently normal, no lesion; 0.5, severely congested serosa and mesenteric vessels engorged with blood; 1, thin walled and friable intestines with small red petechiae (<0.5 cm long); 2, focal necrotic lesions; 3, patches of necrosis (1–2 cm long); and 4, diffused necrosis typical of field cases.
was no evidence of gram-positive rod-shaped organisms attached to intestinal mucosa. Polymorphonuclear (PMN) cell infiltration in the lamina propria was not seen in any of the sections. There was no evidence of coccidial oocysts in any sections examined.

**Discussion**

Glycine is a semi-essential amino acid in young broiler chicks. Three experiments reported in this study support the hypothesis that the dietary glycine concentration is an important determinant of *C. perfringens* growth in the intestinal tract of broiler chickens and thus can predispose birds to NE. The data also indicate that *C. perfringens* growth is not significantly influenced by dietary proline levels tested.

Previous studies in our laboratory demonstrated that diets high in glycine added as animal proteins such as fish meal or gelatin are associated with increased populations of *C. perfringens* (19-21). However, the addition of 40 g/kg of crystalline glycine to the diet had no effect on *C. perfringens* populations in broiler chickens (data not shown). Several studies have reported that crystalline amino acids are absorbed very rapidly in the small intestine (30-32). This rapid absorption might prevent the added glycine from reaching the distal small intestine, making it unavailable to enteric bacteria, including *C. perfringens*.

In Expt. 1, we demonstrated that lipid encapsulation increased glycine concentration in jejenum and ileum of birds, an effect confirmed in Expt. 2 and 3. This suggests that the crystalline glycine was rapidly absorbed in the upper gastrointestinal tract, whereas fat-encapsulated glycine was released slowly along the length of intestine and would therefore be more available to bacteria in the distal bowel. Lipid encapsulation of amino acids has previously been reported for use in aquaculture larval feeds to prevent leaching of amino acids after immersion in water (33,34). However, to our knowledge, lipid-encapsulated amino acids have not been evaluated in poultry.

As evidenced by intestinal lesion scores and high intestinal colonization of *C. perfringens*, most of the birds in this study had a subclinical form of NE, as documented by some earlier researchers (4,12). Decreased growth rate and poor FE have already been reported in broilers with high numbers of *C. perfringens* in the intestinal tract (17,21). The results of our study suggest that birds fed diets containing high levels of glycine will be at greater risk for a clinical outbreak of NE, increased carcass condemnations during processing, and an increased risk of infection in humans.

Previously, Drew et al. (19) documented an increase in *C. perfringens* populations in ileum and cecum of broiler chickens fed fish meal-based diets with 400 g/kg CP. However, there was no difference in *C. perfringens* growth in birds that received soy protein concentrate-based diets containing 230, 315, or 400 g/kg CP. Amino acid analysis of the diets suggested that glycine was in relative excess in the 400-g/kg CP fish meal diet compared with the 400-g/kg CP soy protein concentrate diet. Wilkie et al. (20) compared the effect of various protein sources (animal vs. plant) on intestinal colonization of *C. perfringens* in broiler chickens and reported a significant positive correlation between glycine concentration of the diet and ileal digesta with the number of *C. perfringens* in both ileum and cecum. Earlier, we observed a significant quadratic response for *C. perfringens* growth in cecum of 28-d-old broiler chickens with maximum response at 33.0, 38.9, and 35.1 g/kg dietary glycine concentration with $r^2$ values of 0.95, 0.99, and 0.95 in 3 different experiments (21). In addition, the number of lactobacilli in cecum declined significantly with increasing levels of glycine in these experiments (21). Although glycine levels and *C. perfringens* populations were strongly correlated in this study, gelatin was used as the main source of glycine in the experimental diets and it contains high levels of proline, thereby confounding the results of these experiments.

The exact mechanism(s) by which glycine promotes the intestinal growth of *C. perfringens* in broiler chickens is unclear. Titball et al. (23) documented that growth of *C. perfringens* and production of $\alpha$ toxin are influenced by various amino acids. Glycine accelerated the *C. perfringens* growth (24) and $\alpha$ toxin production required the presence of glycine-containing peptides in defined media (25,26). Putrefactive clostridia and anaerobic Gram-positive cocci are considered important amino acid-fermenting bacteria (35). Earlier, in an in vitro study of amino acid-fermenting bacteria in human large intestine, it was reported that all clostridia were able to metabolize single amino acids, with pairs of amino acids not being essential, although *Clostridium bifermentans* and *Clostridium indolis* were enhanced when provided with Stickland amino acid pairs (36). Some strict anaerobic bacteria have a unique energy-conserving mechanism and they catalyze glycine as substrate using an internal Stickland reaction in which glycine serves as electron donor during oxidation by a glycine cleavage system or as electron acceptor being reduced by glycine reductase. The glycine reductase system is present in several clostridia (*Clostridium sticklandii*, *Clostridium difficile*, *Clostridium litorale*, and *Eubacterium acidaminophilum*) and catalyzes the reductive deamination of glycine to acetylphosphate and ammonia with the generation of ATP from ADP and orthophosphate (37,38). However, *C. perfringens* is not a protein fermentor and this suggests that the effect of higher levels of dietary glycine on intestinal *C. perfringens* populations might be indirect. Because *C. perfringens* is a strong mucolytic bacteria, it enjoys a growth advantage over several other bacteria in the intestinal tract (39).

High-glycine diets may somehow enhance mucus production and secretion through upregulation of mucin gene, thus providing intestinal mucus constantly. It is interesting to note that the differences in *C. perfringens* growth were more pronounced in the cecum than in the ileum despite the fact that glycine concentration might not be different in the cecum (based on results of Expt. 1), which shows that some factors other than glycine might be involved. Clearly, further research is required to elucidate the exact mechanism by which glycine increases *C. perfringens* populations in the intestinal tract of broiler chickens.

In this study, despite inoculation of very high doses of *C. perfringens*, it was not possible to induce NE-specific mortality. Previous controlled studies of chickens challenged with *C. perfringens* have also failed to induce mortality or other signs of NE, even though high *C. perfringens* colonization was reported in the intestinal tract of the birds (3,19,20). In several of the previously described models of clinical NE, birds were coinfected with *Eimeria* spp and *C. perfringens* (8,9,18), but the model described in our study is designed for the subclinical form of the diseases, because lesions are a more sensitive disease indicator than mortality and subclinical NE is more frequent than clinical disease in broiler chickens (4).

In agreement with our previous findings, with increases in dietary glycine concentration there was a corresponding increase in NE lesion scores, although the differences were only significant in Expt. 3. The demonstration of a relation among dietary glycine, *C. perfringens* numbers, and performance data is an important feature of our study. Lesion scores were very similar in birds fed either control2 or G0P4 diets, indicating that dietary...
proline concentrations had no effect on \textit{C. perfringens} growth and NE lesions. Even clinically healthy birds were highly colonized with \textit{C. perfringens}, which confirms the subclinical nature of the disease, because high numbers of \textit{C. perfringens} with macroscopically visible, focal necrotic lesions in the small intestine is a strong indicator of an occurrence of NE (17).

In contrast to some earlier findings, the microscopic lesions in intestine were not conclusive of NE in this study (9,40). Previously, an absence of PMN cells at the site of \textit{C. perfringens} infection has been demonstrated and it was postulated that higher in situ concentration of \textit{C. perfringens} toxins, especially \(\alpha\) toxin, may inhibit PMN cell influx (41). Despite high numbers of \textit{C. perfringens} in the intestinal tract of these birds, the clinical disease could not be produced. It is possible that some other triggering factors might be required to produce a full-blown disease through secretion of \(\alpha\) toxin.

In conclusion, dietary glycine concentration plays an important role in altering \textit{C. perfringens} and lactobacilli populations in the intestinal tract of broiler chickens, whereas proline does not affect this important group of bacteria. Poor FE and reduced growth and sporulation of \textit{C. perfringens} in the postantibiotic era. Animals with a high \textit{C. perfringens} colonization signifies the economic importance of this disease for the poultry producers. Balancing feed composition is probably the most cost-effective prevention and control measure of NE. Knowledge of specific dietary components, such as amino acid profile of different feed proteins and their propensity to cause disease, may aid in the formulation of broiler diets that reduce the risk of NE in the postantibiotic era.

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