Effect of Different Dietary Methionine Sources on Intestinal Microbial Populations in Broiler Chickens

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ABSTRACT Two experiments were conducted to study the effect of various levels of DL-Met or 2-hydroxy-4-methylthiobutanoic acid (MHA-FA) on *Clostridium perfringens* and other intestinal bacteria in broiler chickens. In each experiment, 2 cages of 6 birds (14 d posthatch) were assigned to 1 of 7 different diets in a 2 × 4 factorial arrangement. The main effects were Met source (either DL-Met or MHA-FA) and Met level (0, 0.2, 0.4, or 0.8% DL-Met or 0, 0.227, 0.454, and 0.908% MHA-FA, thus providing 4 corresponding equimolar levels of each Met source). All birds were orally gavaged with a *C. perfringens* type A broth culture on d 1 and on d 14 to 20 and killed on d 28. Intestinal populations of *C. perfringens*, lactobacilli, *Streptococcus* group D, and coliforms were enumerated in the ileum and cecum, and necrotic enteritis intestinal lesions were scored. There was a significant reduction ($P < 0.05$) in *C. perfringens* populations in birds fed either Met source in the cecum (experiment 1) or the ileum and cecum (experiment 2). In experiment 2, the lactobacillus populations were significantly higher ($P < 0.05$) in the ceca of birds receiving 0.8% Met than in the birds given diets with the other levels of Met tested. Significantly lower populations ($P < 0.05$) of coliforms and *Streptococcus* group D were enumerated in the ileum of birds fed the 0.8% Met-supplemented diet than in the other dietary treatments. The effect of Met source on intestinal bacteria was not significant, suggesting that both DL-Met and MHA-FA have similar antibacterial properties. Last, there were no significant differences in intestinal lesion scores or the performance of birds fed different Met sources and concentrations. The results suggest that both DL-Met and MHA-FA may reduce intestinal populations of *C. perfringens* in broiler chickens when used in relatively high concentrations, and may reduce the risk of necrotic enteritis. Thus, feeding low-protein diets supplemented with crystalline amino acids might be beneficial in terms of the growth of various enteric pathogens.

Key words: broiler, DL-methionine, 2-hydroxy-4-methylthiobutanoic acid, necrotic enteritis

2007 Poultry Science 86:2358–2366
doi:10.3382/ps.2007-00133

INTRODUCTION

Clostridial or necrotic enteritis (**NE**) caused by *Clostridium perfringens* type A and C (Ficken and Wages, 1997) is a potentially fatal poultry disease of global significance, and is both an animal welfare and economic problem. Outbreaks of NE are sporadic and may result in high mortality, reduced growth rate, impaired feed conversion, and increased condemnation rates (Lovland and Kaldhusdal, 2001). Necrotic enteritis is estimated to cost the poultry industry as much as US $0.05 per bird, with a total global loss of nearly US $2 billion (Van der Sluis, 2000). However, this might be an underestimate, given the difficulty in diagnosing mild or subclinical forms of NE. In recent years, the banning of prophylactic use of antibiotics in Europe, as well as consumer concerns about their use in other jurisdictions, has stimulated interest in finding alternative management or dietary strategies to control the incidence and severity of this disease in the postantibiotic era.

Clostridium perfringens is frequently found in the intestinal tract of healthy poultry, usually at low levels ($<10^4$ cfu/g of digesta) and is spread in poultry production units and processing plants through feces and intestinal rupture. *Clostridium perfringens* is the main etiological agent of NE, although other cofactors are usually required to precipitate an outbreak of NE (Dahiya et al., 2006). The physical and chemical composition of broiler diets has been reported to have a marked effect on the intestinal microflora of chickens, and it has been shown to have an important impact on the incidence of NE in broiler chickens (Riddell and Kong, 1992; Drew et al., 2004; Dahiya et al., 2006). Dietary cereal grains rich in nonstarch polysaccharides and dietary proteins, especially proteins of animal origin, encourage the development of NE (Rid-

©2007 Poultry Science Association Inc.
Received March 28, 2007.
Accepted July 1, 2007.
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dell and Kong, 1992; Kaldhusdal and Skjerve, 1996; Annett et al., 2002; Wilkie et al., 2005).

Drew et al. (2004) observed a significant increase in *C. perfringens* counts in birds fed a 40% CP fish meal diet; however, *C. perfringens* counts were low in the ileum of birds fed soy protein concentrate-based diets at all levels of CP. A significant positive correlation between the Gly content of the diets and digesta and *C. perfringens* populations in the ileum and cecum of broiler chickens has been reported (Dahiya et al., 2005, 2007; Wilkie et al., 2005). Some in vitro studies have shown an association between certain amino acids and *C. perfringens* growth, α toxin production, or both (Ispolatovskaya, 1971; Muhammed et al., 1975; Nakamura et al., 1978; Titball et al., 1999; Stevens and Rood, 2000). Ispolatovskaya (1971) and Stevens and Rood (2000) reported that Gly or Gly-containing peptides accelerated *C. perfringens* growth and α toxin production in vitro. Although Muhammed et al. (1975) documented that Met was stimulatory for the growth of *C. perfringens* in vitro, previous experiments in our laboratory have demonstrated an antibacterial effect of high concentrations of DL-Met against *C. perfringens* in in vitro experiments (Wilkie et al., 2005; Wilkie, 2006). These researchers reported a significantly reduced growth of *C. perfringens* after a 24-h incubation of mixed bacterial culture in minimal salt media supplemented with a 10 mg/mL solution of DL-Met, compared with unsupplemented media.

Methionine is commonly supplemented as dry DL-Met (99% pure) or as liquid DL-Met hydroxy analog-free acid at concentrations ranging from 0.10 to 0.25% in poultry diets. Based on our preliminary results, we hypothesized that high dietary concentrations of Met are responsible for reduced *C. perfringens* growth in the gastrointestinal tract of broiler chickens and thus may decrease the risk of NE. Hence, the purpose of this experiment was to determine the effect of nutritional and supranutritional levels of 2 Met sources [DL-Met and 2-hydroxy-4-methylthiobutanoic acid (**MHA-FA**)] on intestinal *C. perfringens* and other microbial populations, and on 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 (Canadian Council on Animal Care, 1993).

Experimental Animals, Diets, and Design

In each experiment, a total of 84 one-day-old conventional male broiler chicks (Ross 308) were obtained from a local broiler hatchery (Lilydale Hatchery, Wynyard, Saskatchewan, Canada) and housed randomly in 7 electrically heated battery cages for the first 2 wk of age (12 birds per cage) at the Animal Care Unit, Western College of Veterinary Medicine of the University of Saskatchewan. The birds received a medicated, ideal protein-balanced (3,200 kcal/kg of ME; 1.2% Lys) corn-based starter crumble (Coop Feeds, Saskatoon, Saskatchewan, Canada) for the first 14 d of the experiment (Table 1). On d 14, the birds were weighed and 2 cages of 6 birds each were assigned in a completely randomized design to 1 of the 7 different ideal protein-balanced experimental diets in a 2×4 factorial arrangement. The main effects were Met source (DL-Met or MHA-FA) and Met level (0, 0.2, 0.4, and 0.8% DL-Met or 0.227, 0.454, and 0.908% MHA-FA, thus providing 4 corresponding equimolar levels of each Met source; Tables 2 and 3). The control diet was formulated to contain 23% CP, 1.2% Lys, 0.38% Met, and 3,200 kcal/kg of ME. The control diet was then supplemented with either dry DL-Met or liquid MHA-FA to achieve 4 equimolar levels of each Met source (0, 0.2, 0.4, and 0.8%). The diets met or exceeded the NRC (1994) energy and nutrient requirements for broiler chickens for all other nutrients.

Standard management procedures were followed during this study for both experiments. The research facility

Table 1. Composition of starter diet[1] used in both experiments up to d 14 of age

Ingredient	Inclusion (%)
Soybean meal	40.90
Corn	39.50
Wheat	10.00
Tallow	4.52
Dicalcium phosphate	1.53
Calcium carbonate	1.29
Canola oil	1.00
DL-Met	0.26
Choline chloride	0.10
Vitamin-mineral premix[2]	0.50
Salinomycin	0.06
Bacitracin	0.05
Biocox 120[3]	0.05

[1]Diet was formulated to contain 24.5% CP and meet NRC (1994) requirements for broiler chickens.

[2]Supplied per kilogram of diet: vitamin A, 11,000 IU; cholecalciferol, 2,200 IU; vitamin E, 30 IU; vitamin K, 0.5 mg; vitamin B_{12}, 0.02 mg; thiamine, 1.5 mg; riboflavin, 6 mg; folic acid, 0.6 mg; biotin, 0.15 mg; niacin, 60 mg; pyridoxine, 5 mg; pantothenic acid, 0.02 mg; chloride, 788 mg; sodium, 511 mg; iron, 80 mg; manganese, 21.8 mg; selenium, 0.1 mg; iodine, 0.35 mg; zinc, 100 mg.

[3]Alpharma Inc. (Fort Lee, NJ).

Table 2. Experimental design

Treatment	Met source	Addition of Met source (% of product)	Addition of Met equivalents (%)
1	Control diet	—	—
2	DL-Met	0.200	0.200
3	DL-Met	0.400	0.400
4	DL-Met	0.800	0.800
5	Liquid MHA-FA[1]	0.227[2]	0.200
6	Liquid MHA-FA	0.454[2]	0.400
7	Liquid MHA-FA	0.908[2]	0.800

[1]Liquid MHA-FA = liquid Met hydroxyl analog-free acid.

[2]Based on a liquid MHA-FA content of 88% in the commercial product.

Table 3. Composition of experimental diets used from d 14 to 28 of age in experiments 1 and 2 (% as-is basis)

Item	Control	0.2 DL-Met	0.4 DL-Met	0.8 DL-Met	0.2 MHA-FA[1]	0.4 MHA-FA	0.8 MHA-FA
Ingredient							
Corn	63.75	63.55	63.35	62.95	63.52	63.30	62.84
Meat and bone meal	14.16	14.16	14.16	14.16	14.16	14.16	14.16
Potato protein concentrate	6.88	6.88	6.88	6.88	6.88	6.88	6.88
Soybean meal	6.65	6.65	6.65	6.65	6.65	6.65	6.65
Wheat	5.90	5.90	5.90	5.90	5.90	5.90	5.90
Canola oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Calcium carbonate	0.88	0.88	0.88	0.88	0.88	0.88	0.88
Dicalcium phosphate	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-Thr	0.03	0.03	0.03	0.03	0.03	0.03	0.03
L-Lys HCl	0.10	0.10	0.10	0.10	0.10	0.10	0.10
DL-Met	0.00	0.20	0.40	0.80	0.00	0.00	0.00
MHA-FA	0.00	0.00	0.00	0.00	0.23	0.45	0.91
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin-mineral premix[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Analyzed composition							
CP (%)	23.23	23.37	23.21	23.03	22.83	23.42	23.15
Essential amino acids							
Arg	1.31	1.36	1.28	1.30	1.28	1.32	1.36
His	0.52	0.53	0.52	0.52	0.51	0.52	0.53
Ile	0.90	0.92	0.89	0.90	0.88	0.91	0.91
Leu	2.05	2.06	2.04	2.04	2.02	2.05	2.05
Lys	1.24	1.28	1.24	1.28	1.24	1.28	1.34
MHA-FA	—	—	—	—	0.22	0.50	0.86
Met	0.43	0.63	0.78	1.19	0.43	0.43	0.43
Met + Cys	0.77	0.95	1.12	1.51	0.75	0.75	0.76
Phe	1.08	1.10	1.07	1.07	1.04	1.08	1.09
Thr	0.98	1.03	0.96	1.01	0.95	0.97	1.00
Val	1.11	1.15	1.10	1.12	1.09	1.13	1.13
Nonessential amino acids							
Ala	1.47	1.51	1.47	1.46	1.46	1.48	1.49
Asp	2.11	2.14	2.08	2.11	2.04	2.12	2.12
Cys	0.33	0.33	0.33	0.32	0.32	0.32	0.33
Gly	1.63	1.72	1.60	1.62	1.60	1.63	1.69
Glu	3.56	3.60	3.56	3.56	3.53	3.58	3.57
Pro	1.51	1.56	1.50	1.50	1.50	1.54	1.55
Ser	1.15	1.16	1.14	1.16	1.12	1.17	1.15

[1]2-Hydroxy-4-methylthiobutanoic acid.

[2]Supplied per kilogram of diet: vitamin A, 11,000 IU; cholecalciferol, 2,200 IU; vitamin E, 30 IU; vitamin K, 0.5 mg; vitamin B_{12}, 0.02 mg; thiamine, 1.5 mg; riboflavin, 6 mg; folic acid, 0.6 mg; biotin, 0.15 mg; niacin, 60 mg; pyridoxine, 5 mg; pantothenic acid, 0.02 mg; chloride, 788 mg; sodium, 511 mg; iron, 80 mg; manganese, 21.8 mg; selenium, 0.1 mg; iodine, 0.35 mg; zinc, 100 mg.

was thoroughly cleaned and disinfected prior to bird placement. The battery cages were arranged in 4 tiers with wire floors and were equipped with external feed and water troughs. The lighting schedule was 16L:8D throughout the experiment, with controlled temperature and humidity. Room temperature was maintained according to industry standards. None of the experimental diets contained antibiotics or coccidiostats, and diets were not pelleted. Throughout the experimental period in each experiment, birds had ad libitum access to food and water. Feed consumption and BW for each cage were recorded for the period of d 14 to 21 and d 21 to 28 in each experiment to calculate feed conversion. When calculating feed conversion, the BW of dead birds was taken into account. Amino acid analysis of the different diets was performed by the Degussa Corporation (Degussa Canada Inc., Burlington, Ontario, Canada).

C. perfringens Challenge Model

The *C. perfringens* challenge model was based on the model originally developed by Dahiya et al. (2005), with some modifications. Briefly, an avian *C. perfringens* field strain isolated from a clinical case of NE was obtained from Manuel Chirino, College of Veterinary Medicine, University of Saskatchewan, and characterized by the PCR technique as a type A toxin producer. The organism was cultured anaerobically on BBL blood agar base (Becton, Dickinson and Co., Sparks, MD) containing 5.0% sheep blood and 100 mg/L of neomycin sulfate (The Upjohn Company) for 18 h at 37°C, and then aseptically inoculated into either brain heart infusion (Difco Labs, Detroit, MI; experiment 1) or cooked meat medium (Difco Labs; experiment 2) and incubated anaerobically overnight (experiment 1) or for 8 h (experiment 2) at 37°C. All birds were orally challenged in the crop with this actively growing culture of *C. perfringens* with 0.5 mL on d 1 and 1.0 mL on d 14 to 20, inclusive, by 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 4.51×10^4 to 3.73×10^6 cfu/mL.

Pathological Examination

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 the experiments were necropsied to determine the cause of death. On d 28, the surviving chickens were killed by cervical dislocation, weighed, and necropsied immediately. Intestinal tracts were removed and intestinal lesions were scored blindly according to the method of Truscott and Al-Sheikhly (1977), with slight modifications, on a scale of 0 to 4 as described previously by Dahiya et al. (2005). Following postmortem examination, if the score was ≥1 then a 1.5- to 2.0-cm-long piece of intestinal tissue with the gross lesion was collected in phosphate-buffered formaldehyde solution and processed routinely for paraffin embedding, sectioned at approximately 5 μm, and stained with hematoxylin and eosin.

Bacterial Enumeration

As described above, on d 28, birds were selected at random from each pen, weighed, and killed by cervical dislocation and their intestinal tracts were removed. Samples of fresh digesta (0.1 to 0.2 g) from the ileum (Meckel's diverticulum to 1 cm proximal to the ileocecal junction) and ceca were collected aseptically in preweighed 15-mL sterilized plastic tubes containing 1 mL of 0.1% sterile peptone buffer with 5 g/L of Cys hydrochloride (Sigma Chemical Co., St. Louis, MO). The digesta samples were pooled from 2 birds from each cage. The samples were immediately placed on ice and kept there until plated, within 3 h of collection. The samples were weighed and diluted in peptone water to an initial 10^{-1} dilution. Tenfold dilutions were spread in duplicate with an automated spiral plater (Autoplate, Spiral Biotech Inc., Bethesda, MD) on BBL blood agar base (Becton, Dickinson and Co.) containing 5% sheep blood and 100 mg/L of neomycin sulfate (The Upjohn Company) for *C. perfringens* enumerations. In addition, all the digesta samples were cultured on de Man, Rogosa, Sharpe agar (Becton, Dickinson and Co.), MacConkey's agar (Becton, Dickinson and Co.), and bile esculin agar (Becton, Dickinson and Co.) for enumeration of lactobacilli, coliforms, and *Streptococci* group D, respectively. The plates were incubated at 37°C for 16 to 24 h anaerobically for *C. perfringens* and lactobacilli and aerobically for coliforms and *Streptococcus* group D bacteria. 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 Inc., Nepean, Ontario, Canada), and examined microscopically to confirm them as *C. perfringens*. Counts were expressed as $\log_{10}$ cfu per gram of intestinal contents.

Statistical Analysis

The data were analyzed by the GLM procedure of SPSS (v. 12.0, SPSS Inc., Chicago IL) as a 2 × 4 factorial (2 Met sources, each with 4 levels). All the effects were considered as fixed, and the interactions between Met source and level were used in the model, with pen as the experimental unit. There was no pen effect on any of the measured parameters in either experiment. The digesta samples for bacterial enumeration were pooled from 2 birds from each pen, resulting in 6 observations for each diet in each experiment. The treatment means were compared by using the Ryan-Einot-Gabriel-Welch multiple *F*-test, and comparisons were deemed significant at $P < 0.05$.

RESULTS

The CP and amino acid composition of the experimental diets used in this study from d 14 to 28 of age is shown in Table 3. The dietary CP, DL-Met, and MHA-FA concentrations were very close to our planned levels. All of the other amino acids and MHA-FA were in similar concentrations in all experimental diets in both experiments.

In both experiments, some of the birds initially became dull and depressed, and had abnormally wet droppings after the *C. perfringens* challenge was initiated on d 14. During the course of this study, 2 birds died in experiment 1 (1 each from the control and 0.4% DL-Met-supplemented groups) and 3 birds died in experiment 2 (1 each from the control, 0.2, and 0.8% MHA-FA-supplemented groups) of unknown causes. The majority of dead birds were in good condition and had no typical field-type gross lesions of NE in either the intestine or any other organ that might have caused the death. Only 1 bird had a distended jejunum and ileum with a thin and friable intestinal wall, and the lumen was filled with gas and dark brown fluid content. There were few petechial hemorrhages in the distal jejunum and proximal ileum in the remaining dead birds, whereas the surviving birds displayed no obvious signs of morbidity at 7 to 10 d postchallenge.

The average feed consumption, BW gain, and feed conversion for the periods of d 14 to 21 and d 21 to 28 of age were not significantly different among various dietary treatments in any of the experiments (data not shown). In addition, there was no significant interaction between Met source and level for various performance parameters.

On d 28 of age, the populations of all 4 bacterial species enumerated in this study (*C. perfringens*, lactobacilli, *Streptococcus* group D, and coliforms) were higher in the cecum than in the ileum of broiler chickens. There were no significant differences in the growth of various bacterial species in the intestinal tract of broiler chickens fed 2 different Met sources, whereas Met concentration had a significant effect on various bacterial species either in the ileum or cecum, or both (Tables 4 and 5). There was no significant interaction between Met source and Met concentration for various bacterial populations in either the ileum or cecum in either experiment. The only exception was *C. perfringens* growth in the ileum and cecum in experiment 2, where there was a significant interaction between Met source and level. There was a decrease in the numbers of *C. perfringens* with an increase in dietary DL-Met in both the ileum and cecum, whereas *C. perfringens* counts

Table 4. Effect of different Met sources or levels on various bacterial populations in the ileum of broiler chickens on d 28 of age in experiments 1 and 2[1]

	Clostridium perfringens		Lactobacilli		*Streptococcus* group D		Coliforms	
Item	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Met level (%)								
0.0	3.08	4.86[a]	7.66	8.68	6.02	6.66[a]	4.63	6.66[a]
0.2	1.28	3.64[b]	7.67	8.44	5.50	6.03[a]	4.60	5.91[b]
0.4	0.60	3.38[bc]	7.69	8.19	5.41	6.03[a]	4.89	5.79[b]
0.8	1.34	2.95[c]	7.67	8.52	5.66	5.51[b]	3.76	5.09[c]
Pooled SEM	1.171	0.916	0.018	0.252	0.306	0.553	0.684	0.762
Met source (%)								
DL-Met	1.37	3.47	7.67	8.33	5.60	6.07	4.52	5.80
MHA-FA[2]	1.35	3.61	7.68	8.52	5.59	5.86	4.37	5.70
Pooled SEM	0.020	0.020	0.016	0.142	0.037	0.248	0.092	0.155
Source of variation (*P*-value)								
Level	0.103	<0.001	0.999	0.611	0.052	0.003	0.576	<0.001
Source	0.970	0.878	0.910	0.537	0.769	0.135	0.877	0.344
Level × source	0.494	0.001	0.857	0.940	0.074	0.350	0.497	0.170

[a-c]Means with different superscripts within a column (within an experiment) differ ($P < 0.05$).

[1]Means are $\log_{10}$ cfu/g of intestinal contents, n = 6 for Met level and n = 21 for Met source.

[2]2-Hydroxy-4-methylthiobutanoic acid.

Table 5. Effect of different Met sources or levels on various bacterial populations in the ceca of broiler chickens on d 28 of age in experiments 1 and 2[1]

	Clostridium perfringens		Lactobacilli		*Streptococcus* group D		Coliforms	
Item	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Met level (%)								
0.0	5.22[a]	5.94[a]	8.51	8.67[b]	7.01[a]	6.96	6.24	7.21
0.2	1.87[b]	4.30[b]	8.57	8.77[b]	6.21[b]	7.21	5.00	7.26
0.4	1.49[b]	4.54[b]	8.87	8.90[b]	6.86[ab]	7.12	5.02	7.16
0.8	3.22[ab]	4.26[b]	8.66	9.43[a]	6.48[ab]	7.11	5.96	7.13
Pooled SEM	1.929	0.858	0.202	0.446	0.460	0.120	0.797	0.079
Met source (%)								
DL-Met	2.73	4.57	8.72	8.97	6.73	7.19	5.62	7.21
MHA-FA[2]	2.52	4.62	8.62	8.98	6.45	7.05	5.29	7.16
Pooled SEM	0.104	0.037	0.000	0.085	0.236	0.215	0.263	0.084
Source of variation (*P*-value)								
Level	0.005	<0.001	0.493	0.008	0.037	0.581	0.402	0.798
Source	0.863	0.840	0.996	0.567	0.206	0.058	0.642	0.389
Level × source	0.338	0.021	0.163	0.192	0.686	0.121	0.964	0.373

[a,b]Means with different superscripts within a column (within an experiment) differ ($P < 0.05$).

[1]Means are $\log_{10}$ cfu/g of intestinal contents, n = 6 for Met level and n = 21 for Met source.

[2]2-Hydroxy-4-methylthiobutanoic acid.

were higher in the ileum (3.38 vs. 3.90 $\log_{10}$ cfu/g of wet digesta) and cecum (3.98 vs. 4.98 $\log_{10}$ cfu/g of wet digesta) when dietary MHA-FA was increased from 0.2 to 0.4%. However, there were no significant differences in *C. perfringens* counts between DL-Met and MHA-FA at the 0.2 or 0.4% inclusion rates.

In experiment 2, significantly higher numbers of *C. perfringens* were observed in the ileum of birds fed the control diet as compared with birds given Met-supplemented diets. In addition, *C. perfringens* populations in the ileum decreased significantly with an increase in Met supplementation from 0.2 to 0.8%. In experiment 1, *C. perfringens* growth in the ileum was not significantly different among various dietary treatments. In experiment 1, the ceca of birds fed either 0.2 or 0.4% Met contained significantly lower *C. perfringens* counts than the ceca of birds in the control diet with no supplemental Met. The control diet supported a significantly higher growth of *C. perfringens* than the Met-supplemented diets in experiment 2. An important point to note is that the *C. perfringens* populations were generally higher in both the ileum and cecum in experiment 2 compared with experiment 1.

The lactobacillus populations were not significantly altered in the ileum of broiler chickens fed different levels or sources of Met in the 2 experiments (Table 4). Similarly, there were no significant differences in lactobacilli growth in the cecum in experiment 1. However, in experiment 2, the lactobacillus populations were significantly higher in the ceca of birds receiving 0.8% Met than in those of birds given diets with the other levels of Met tested (Table 5).

In experiment 2, *Streptococcus* group D populations were significantly lower in the ileum of birds receiving 0.8% Met-supplemented diets compared with those given the control, 0.2, or 0.4% Met-supplemented diets. We observed a significant difference in *Streptococcus* group D growth in the ceca of birds receiving the control diet and the 0.2% Met-supplemented diet in experiment 1, whereas there were no significant differences in the ceca of birds in experiment 2.

In experiment 2, the coliform counts were significantly higher in the ileum of broiler chickens that were given the control diets with no added Met compared with birds receiving the rest of the experimental diets with different concentrations of Met. Methionine source or level had no significant impact on coliform counts in the cecum in either experiment 1 or 2 (Table 5). The coliform counts were substantially lower than those of lactobacilli and streptococci in the intestines of chickens on d 28 of age.

The mean NE lesion score of chickens fed different experimental diets and killed on d 28 is depicted in Figure 1. Dietary Met source or concentration had no significant effect on NE-specific intestinal lesion scores in either experiment. Irrespective of the dietary treatment, a large number of birds had a thin and friable intestinal wall, with congested serosa with blood-engorged mesenteric vessels. Some birds had focal patches of hemorrhagic lesions in various intestinal regions, more frequently in the distal jejunum, proximally ileum, and cecal tonsils.

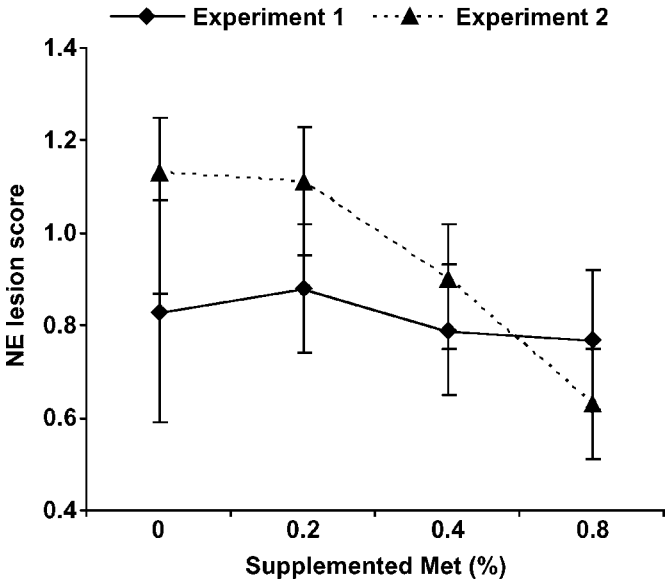

Figure 1. Mean necrotic enteritis (NE) lesion scores in experiments 1 and 2 in 28-d-old broiler chickens given experimental diets (d 14 to 28) containing different levels or sources of Met. Bars represent mean ± SEM, n = 12. Lesion were scored on a 0 to 4 scale, where 0 = no intestinal lesion (apparently normal); 0.5 = severely congested serosa and mesentery engorged with blood; 1 = thin-walled and friable intestines with small red petechiae (>5); 2 = focal necrotic lesions; 3 = patches of necrosis (1 to 2 cm long); and 4 = diffused necrosis typical of field cases.

However, typical field-type lesions specific to NE were not observed in any of the birds in either experiment. Although Met source or concentration had no significant effect on intestinal lesion scores, there was a trend of decreasing lesions with an increase in Met concentration, which corresponds well with *C. perfringens* colonization in these birds.

Histological examination of formalin-fixed intestinal tissues from 28-d-old broiler chickens euthanized in both experiments revealed no lesions that were highly suggestive of NE. The lesions included only slight edema and diffuse hemorrhages in the lamina propria in some sections. No visible signs of necrosis or desquamation of epithelial cells in the villi were reported in either experiment. There was no evidence of gram-positive, rod-shaped organisms attached to the intestinal mucosa. Polymorphonuclear cells (**PMN**) infiltration in the lamina propria was not seen in any of the sections. We found no evidence of coccidial oocysts in any of the sections examined.

DISCUSSION

Evidence suggests that the amino acid composition of various protein sources is an important determinant of intestinal microbial growth in broiler chickens (Dahiya et al., 2006). Excessive concentrations of some amino acids might have a toxic effect on some bacterial species. Wilkie (2006) demonstrated a reduced growth of *C. perfringens* in the presence of Met in vitro. In one of his studies, he demonstrated that *C. perfringens* counts were virtually reduced to zero in minimal salt media supplemented with

10 mg/mL of DL-Met after a 24-h incubation. Hence, the current study was designed to determine whether 2 commonly used Met sources would have similar antibacterial effects against *C. perfringens* in vivo.

The overall performance of the birds was relatively poor in both experiments, which might be because the birds were under stress from the *C. perfringens* challenge and had high *C. perfringens* populations in their intestinal tract, which is evident from Tables 4 and 5. The average feed consumption and BW gain were lower and the feed:gain ratio was comparatively poor in experiment 2 during the 21- to 28-d period, which might be related to the higher *C. perfringens* counts in experiment 2 compared with experiment 1. As evidenced by the intestinal lesion scores and high intestinal colonization of *C. perfringens*, most of the birds in the present study had subclinical NE, as documented by Lovland and Kaldhusdal (2001). Decreased growth rate and poor feed conversion efficiency have been reported previously in broilers having high numbers of *C. perfringens* in the intestinal tract (Stutz and Lawton, 1984; Kaldhusdal and Hofshagen, 1992; Dahiya et al., 2005, 2007).

Numerous studies have been conducted to compare the efficacy of DL-Met and liquid MHA-FA in broiler chickens (see, for example, Rostagno and Barbosa, 1995; Lemme et al., 2002). Some of these studies have been inconsistent in the value assigned to the efficacy of MHA-FA for a number of reasons (Maenz and Engele-Schaan, 1996; Drew et al., 2003). Drew et al. (2003) compared the apparent absorption of ^{3}H-labeled L-Met with MHA-FA in germ-free and conventionally reared broiler chickens and observed a significantly lower residual MHA-FA in the distal ileum of germ-free birds than in conventional birds, in which there was no difference in residual Met level. Questions remain regarding the effect of these 2 Met sources on the intestinal microbial ecology of the birds. To our knowledge, this is the first study in which the direct impact of these 2 Met sources on the intestinal microflora of broiler chickens has been examined.

Clostridium perfringens populations were higher in the ileum and cecum of birds in experiment 2 than in experiment 1. This result might be because in experiment 2, *C. perfringens* was cultured in cooked meat medium, which supports *C. perfringens* growth and α toxin production much better than brain heart infusion medium, which was used in experiment 1 (J. P. Dahiya, personal communication). In the present study, we observed a significant reduction in *C. perfringens* growth with Met supplementation in the cecum (experiment 1), or in both the ileum and cecum (experiment 2). However, there were no significant differences between the 2 Met sources. We could find no literature on the toxic effects of high concentrations of Met on *C. perfringens* in vivo. Earlier, Muhammed et al. (1975) reported that Ala, Asp, and Met were stimulatory for the growth and sporulation of *C. perfringens* in vitro, but we cannot extrapolate the results from in vitro to in vivo because the conditions are entirely different in the gastrointestinal tract. There are differences in amino acid and vitamin requirements among various strains of *C. perfringens* (Fuchs and Bonde, 1957). Fuchs and Bonde also reported a significant increase in *C. perfringens* growth in the presence of Gly, Lys, and Ser and proved that not only individual amino acids but also the balance of amino acids is important for maximum growth of *C. perfringens*. There might be antagonism between amino acids (i.e., the action of certain amino acids being prevented by several other amino acids). Previously, we found that the amino acid Gly supports *C. perfringens* growth and α-toxin production both in vitro and in vivo (Dahiya et al., 2005, 2007; Wilkie et al., 2005).

The Met levels used in this study ranged to 2 to 4 times higher than those commonly used in commercial poultry diets. The supranutritional concentrations of Met were associated with reduced *C. perfringens* growth in broiler chickens; however, it might not be a commercially viable method of controlling NE because of the high cost of crystalline Met. The mode of action by which Met influences the intestinal populations of these important groups of bacteria in broiler chickens is unclear. Not all bacteria have the ability to utilize DL-Met or MHA-FA. Because *C. perfringens* is strictly dependent on carbohydrates, the effect of higher levels of dietary Met on intestinal *C. perfringens* populations might be indirect.

In experiment 2, we observed a significant elevation in lactobacillus population in the cecum of birds fed 0.8% Met-supplemented diets compared with the rest of the diets. In contrast to this, *Streptococcus* group D populations were decreased with Met supplementation in the ileum (experiment 2) and cecum (experiment 1). Hofshagen and Kaldhusdal (1992) reported a higher population of lactobacilli in the intestinal tract of broiler chickens fed corn-based diets and hypothesized that higher lactobacillus colonization impeded the development of NE. This effect might be indirect through the inhibition of *C. perfringens* colonization. We observed substantially lower populations of coliforms than lactobacilli and streptococci in the intestines of chickens, which is consistent with the findings of Barnes et al. (1972) and Stutz et al. (1983) and opposed to the findings of Hofshagen and Kaldhusdal (1992), who reported similar levels of these bacterial species. This discrepancy may be attributed to variations in bacteriological isolation procedures, feed composition, feed antibiotics, or environmental conditions among the studies.

Hegedus et al. (1993) documented that some lactic acid-producing bacteria (*Lactobacillus plantarum, Leuconostoc mesenteriodes, Lactobacillus casei*) have the ability to utilize DL-Met for their growth. Lactic acid bacteria have been characterized for their antagonistic action against *Salmonella* and *Campylobacter* in poultry (Gusils et al., 2003; Lan et al., 2003). Fukata et al. (1991) reported that the pathogenic effect of *C. perfringens* could be reduced by feeding chicks a monoflora of *Lactobacillus acidophilus* or *Streptococcus faecalis*. It is also possible that the proteins or amino acids are serving as an energy or nitrogen source for other bacterial species, which in turn modify the intestinal environment in a favorable way for lactobacillus proliferation.

In both experiments, despite inoculation of very high doses of *C. perfringens*, the mortality was low (2.38 and 3.57%, respectively), and necropsy examinations revealed that the deaths were not NE specific. This is in agreement with some previous studies in which chickens challenged with *C. perfringens* failed to induce mortality or other signs of NE, even though high colonization of *C. perfringens* was reported in the intestinal tract of the birds (Craven, 2000; Pedersen et al., 2003). In contrast to this, Al-Sheikhly and Truscott (1977) and Vissiennon et al. (2000) were able to induce various pathological changes and mortality in chickens when inoculated orally with *C. perfringens* directly into the duodenum. It is therefore possible that some of the vegetative cells are inactivated by the acidic pH in the gizzard when given orally.

In the present study, we observed a decreasing trend of NE intestinal lesions with a corresponding increase in dietary Met concentration. The demonstration of a relationship between NE lesion scores, performance data, and *C. perfringens* numbers is an important feature of both experiments. Al-Sheikhly and Truscott (1977) and Vissiennon et al. (2000) suggested that high *C. perfringens* populations and slight intestinal damage were apparently necessary for disease production when a broth culture was used. Earlier, Tanya et al. (2005) demonstrated a reduction in intestinal NE lesion scores in broiler chickens fed low-protein diets (CP 18%).

In contrast to some earlier findings, the microscopic lesions in the intestine were not conclusive of NE in the current study (Shane et al., 1985; Kaldhusdal et al., 1995). The presence of slight edema and hemorrhages in the lamina propria was observed in many birds. However, desquamated epithelial cells and PMN cells were not detected in any of the sections. Bryant et al. (1993) and Stevens et al. (1997) also demonstrated an absence of PMN cells at the site of *C. perfringens* infection. In the absence of a host response (suppression of PMN influx), clostridia proliferates rapidly, leading to local accumulation of toxins. Higher in situ concentrations of *C. perfringens* toxins, especially α toxin, further inhibit PMN influx and reach concentrations sufficient to cause membrane destruction (Stevens and Rood, 2000). Hence, in spite of high numbers of *C. perfringens* in the intestinal tract of these birds, the clinical disease could not be produced.

The results of the present study demonstrated that both Met sources might have some antibacterial effect against *C. perfringens*. Thus, it might be possible to inhibit *C. perfringens* growth in the intestinal tract of broiler chickens and prevent the occurrence of NE outbreaks through supplementation of low-protein diets with relatively high amounts of Met. Feeding low-CP diets supplemented with crystalline amino acids might be beneficial in terms of the growth of various enteric pathogens. Because there are some limitations to the culture-based methods for bacterial enumeration used in the present study, it would be interesting to use culture-independent approaches, such as denaturing gradient gel electrophoresis, in studying a wide group of microbiota with respect to different Met sources. Further studies to determine which bacterial species are involved in competition with the host for these 2 Met sources would be interesting.

ACKNOWLEDGMENTS

The authors acknowledge the financial support of the Natural Sciences and Engineering Research Council of Canada (Ottawa, Ontario, Canada) and Degussa Corporation. The technical assistance of Darryl Wilkie and Jason Marshall is greatly appreciated.

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