



Original Research Article

The role of supplemental glycine in establishing a subclinical necrotic enteritis challenge model in broiler chickens



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ABSTRACT

Subclinical necrotic enteritis (NE) is an economically important disease in the broiler industry. With the move towards removal of antibiotics from feeds, solutions to control subclinical NE are desperately being sought. Dietary glycine has been shown to promote proliferation of *Clostridium perfringens* (Cp) and may thus be useful to include in a NE challenge model. A study was conducted to evaluate the effect of increased dietary glycine levels on subclinical NE. A $2 \times 2 \times 2$ factorial arrangement of treatments was carried out using day-old male Ross 308 chicks ($n = 624$) allocated to 48 floor pens with 8 treatments of 6 replicates with 11 birds per treatment. Factors were: Cp challenge (C– or C+), *Eimeria* spp. challenge (E– or E+), and dietary glycine in the grower diet (0 or 10 g/kg). Birds had higher FCR when challenged with *Eimeria* ($P < 0.01$) or Cp ($P < 0.05$) on d 24 or Cp on d 35 but FCR was lower when fed glycine on d 24 ($P < 0.01$). Supplementation of glycine reduced feed intake on d 24 and increased weight gain on d 35 ($P < 0.05$). A Cp \times *Eimeria* \times glycine interaction ($P < 0.05$) showed a higher jejunal lesion scores in birds challenged with a combination of Cp and glycine compared with those with *Eimeria* and glycine or the unchallenged birds. Lesion score interactions between Cp and glycine ($P < 0.05$) in the ileum and Cp and *Eimeria* in the duodenum ($P < 0.05$) and ileum ($P < 0.05$) illustrated higher lesion scores in birds challenged with Cp without *Eimeria* or glycine compared to those not challenged with Cp. This study suggests that using glycine can partially replace *Eimeria* in a subclinical NE challenge model in promoting the intestinal lesions but not impairing chicken performance.

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1. Introduction

Necrotic enteritis (NE) is a costly bacterial disease for the broiler chicken industry (Van der Sluis, 2000). The clinical form of NE causes high mortality up to 30% in an infected flock (Van Immerseel et al., 2009), but subclinical NE predominately occurs in the poultry industry and causes the most devastating losses to profitability as subclinical NE results in poor feed efficiency and can persist in flocks without detectable clinical manifestations, so

often remains untreated (Skinner et al., 2010). A better understanding of this disease is required in order to develop strategies for its control and prevention. A reproducible, reliable and well-characterized challenge model is required to successfully conduct research on subclinical NE. Challenge models for NE often perform inconsistently, varying between clinical and subclinical form of the disease. *Eimeria* co-infection arguably plays a key role in this lack of consistency (Shojadoost et al., 2012). This suggests a subclinical NE challenge model may be more reliable if *Eimeria* is omitted. *Clostridium perfringens* (Cp) is the main causative agent of NE. It is an opportunist spore-forming organism that is prevalent in the gut of healthy chickens (Kondo, 1988). It is therefore imperative that a subclinical NE model be established that can be induced under laboratory conditions consistently. Under favorable conditions, *Clostridia* spores germinate and release toxins such as necrotic enteritis B-like toxin that are cytotoxic to enterocytes thus playing a significant role in NE prevalence and severity (Burns et al., 2010; Keyburn et al., 2010). Germination of Cp requires the

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spore cortex to be removed by the spore cortex-lytic enzyme (SCLE), which is coded by *SleC* (Paredes-Sabja et al., 2008, 2009; Adams et al., 2013). Glycine forms a conjugated compound with bile salts that plays a critical role in Cp germination (Miyata et al., 1997) and interacts with *SleC* gene that is involved in the germination patterns of *Clostridia* (Sorg and Sonenshein, 2008). There is direct evidence that feeding broilers diets high in glycine increases the intestinal Cp number. Kocher (2003) found a strong relationship between dietary fish meal that has a high glycine content, Cp proliferation and severity of NE. Glycine induces NE through its direct impact on the intestinal population of Cp, suggesting that glycine could potentially play a significant role in a NE challenge model.

The aim of this study was to investigate the roles of *Eimeria* and dietary glycine in inducing a subclinical NE challenge, and to test if a successful subclinical NE model could be achieved without the presence of *Eimeria* by rising the glycine level in the diet.

2. Materials and methods

This study was conducted at University of New England, Armidale, New South Wales, Australia. All experimental procedures and protocols involved in this study were reviewed and approved by the Animal Ethics Committee of the University of New England. Birds were cared for according to the Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013).

2.1. Experimental design, diet and bird husbandry

A total of 528 day-old male broiler chickens (Ross 308) were obtained from a local hatchery (Baiada Country Road Hatchery, Tamworth, NSW, Australia). Chicks were vaccinated against Marek's disease, infectious bronchitis and Newcastle disease at hatchery. From d 0 to 9, chicks were housed and reared in an environmentally controlled room bedded with fresh wood shaving with *ad libitum* access to water and a common starter diet. On d 9, after 3 h fasting, birds were weighed and evenly distributed into appropriate pens to ensure uniform initial starting pen weights. A floor pen system (approx. area 120 cm × 75 cm per pen) with fresh wood shavings was used. Birds were assigned to a 2 × 2 × 2 factorial arrangement of treatments, with 6 replicate pens per treatment and 11 birds per pen. The factors were: Cp challenge (C+ or C-), *Eimeria* spp. challenge (E+ or E-), and supplemental glycine (0 or 1%) in the grower diet from d 9 to 24 (Redox Pty Ltd, Brisbane, Queensland, Australia). The diets were wheat, soybean, meat meal and canola meal based and were formulated based on Aviagen (2012) nutritional specification guidelines and fed as pellets. Birds had *ad libitum* access to feed and water throughout the experiment.

The facility was disinfected prior to bird's arrival. Lighting regime and rearing temperature followed the breeder guideline during the experiment (Aviagen, 2014). Pen weight and cumulative pen feed intake were recorded on d 9, 24 and 35 and used to calculate mean individual bird weight, feed intake and FCR (corrected for mortality and sampled birds).

2.2. Coccidiosis challenge

On d 9, each bird in the *Eimeria*-challenged group was orally administered an attenuated vaccine strain of *Eimeria*. Each 1 mL gavage included PBS suspension of approximately 5,000 oocysts each of *E. acervulina*, and *E. maxima*, and 2,500 sporulated oocysts of *E. brunetti* (Bioproperties Pty Ltd., Glenorie, NSW, Australia). In the unchallenged groups, 1 mL of sterile PBS was administered as the control treatment.

2.3. NE challenge and lesion scoring

On d 14 and 15, Cp-challenged birds were inoculated per os with 1 mL of Cp suspension at a concentration of 10⁸ to 10⁹ cfu/mL using a primary poultry isolate of *C. perfringens* type A strain EHE-NE18 (CSIRO Livestock Industries, Geelong, Australia). Preparation of Cp in the current study was followed the method as described by Wu et al. (2010). Birds in the unchallenged groups received 1 mL of sterile inoculum.

On d 16, 2 birds were randomly selected and euthanized by cervical dislocation. The entire length of the section of small intestine (duodenum, jejunum and ileum) of all sampled birds underwent a lesion scoring process, based on a previously reported lesion scoring system that ranges from 0 to 4 (Prescott et al., 1978; Broussard et al., 1986).

2.4. Statistical analyses

Statistical analysis was conducted using IBM SPSS Statistics package version 22 (IBM Corporation, Armonk, NY, United States). The main effects and interactions between glycine supplement, *Eimeria* challenge and Cp challenge on bird performance were examined by analysis of variance using the General Linear Model. Lesion score data were analyzed using the nonparametric Kruskal–Wallis test as they were not normally distributed. Treatment means were separated using the Tukey multiple range test where appropriate. Statistical significance was declared at $P < 0.05$.

3. Results

3.1. Necrotic enteritis symptoms of birds and mortality

Within hours following the induction of the Cp challenge, infected birds displayed NE symptoms such as depression, ruffled feathers, loss of appetite and closed eyes. These symptoms were pronounced for approximately 6 h and then gradually dissipated, with birds started to recover by 12 h following the Cp challenge. This indicates that NE symptoms tend to be transient. No mortality related to NE occurred within 72 h after Cp challenge.

3.2. Performance

Performance parameters measured on d 24 and d 35 are presented in Table 1. No significant Cp × glycine or *Eimeria* × glycine interactions, or three-way interaction between Cp × glycine × *Eimeria*, were observed on bird performance at either d 24 or 35. A Cp × *Eimeria* interaction ($P < 0.05$) was observed for weight gain on d 24, showing that birds challenged with Cp or *Eimeria* alone had no effect on weight gain, but when exposed in combination, weight gain was significantly reduced. Feed intake was lower in birds fed the diet containing 1% additional glycine ($P < 0.05$) which resulted in lower FCR ($P < 0.01$). FCR was significantly higher in the birds challenged with *Eimeria* ($P < 0.01$) or Cp ($P < 0.01$) at d 24 and Cp ($P < 0.05$) at d 35 compared to the unchallenged birds.

3.3. Intestinal lesion score

Intestinal lesion scores in the duodenum, jejunum and ileum measured on d 16 are presented in Table 2. Three-way interactions between Cp × glycine × *Eimeria* ($P < 0.05$) were observed for lesion scores in the jejunum. This showed that lesions were more prevalent and severe in birds challenged with a combination of Cp and glycine compared with birds challenged with *Eimeria* and glycine or with no challenge. There were Cp × *Eimeria* interactions for lesion

Table 1
Effect of *Clostridium perfringens* (Cp) challenge, *Eimeria* spp. and dietary glycine on performance of broilers from d 9 to 24 and from d 9 to 35.

Treatment means	d 9 to 24			d 9 to 35		
	Weight gain, g/bird	Feed intake, g/bird	FCR	Weight gain, g/bird	Feed intake, g/bird	FCR
<i>Cp</i> × <i>Eimeria</i>						
C− E−	1,040 ^a	1,386	1.333	2,185	3,369	1.542
C− E+	1,045 ^a	1,425	1.364	2,243	3,528	1.573
C+ E−	1,041 ^a	1,422	1.367	2,171	3,547	1.633
C+ E+	1,001 ^b	1,432	1.433	2,233	3,635	1.629
<i>Cp</i> × Glycine						
C− G−	1,039	1,422	1.370	2,201	3,443	1.564
C− G+	1,046	1,388	1.327	2,228	3,455	1.551
C+ G−	1,007	1,441	1.434	2,144	3,479	1.624
C+ G+	1,035	1,413	1.367	2,259	3,703	1.637
<i>Eimeria</i> × Glycine						
E− G−	1,038	1,424	1.373	2,146	3,365	1.571
E− G+	1,042	1,383	1.328	2,210	3,552	1.604
E+ G−	1,007	1,439	1.431	2,198	3,557	1.618
E+ G+	1,039	1,418	1.366	2,278	3,606	1.584
Main effects						
<i>C. perfringens</i>						
Cp−	1,042 ^a	1,406	1.349 ^b	2,214	3,449	1.558 ^b
Cp+	1,021 ^b	1,427	1.400 ^a	2,202	3,591	1.631 ^a
<i>Eimeria</i>						
E−	1,040	1,404	1.350 ^b	2,178	3,458	1.587
E+	1,023	1,428	1.399 ^a	2,238	3,582	1.601
Glycine ¹						
G−	1,023	1,432 ^a	1.402 ^a	2,172 ^b	3,461	1.594
G+	1,040	1,401 ^b	1.347 ^b	2,244 ^a	3,579	1.594
SEM	5.4	7.8	0.010	17.1	44.6	0.015
<i>P</i> -value						
Cp	0.030	NS	0.006	NS	NS	0.019
<i>Eimeria</i>	NS	NS	0.009	NS	NS	NS
Glycine	NS	0.033	0.003	0.038	NS	NS
Cp × <i>Eimeria</i>	0.025	NS	NS	NS	NS	NS
Cp × Glycine	NS	NS	NS	NS	NS	NS
<i>Eimeria</i> × Glycine	NS	NS	NS	NS	NS	NS
Cp × <i>Eimeria</i> × Glycine	NS	NS	NS	NS	NS	NS

NS = not significant.

^{a, b}Means not sharing the same superscripts are significantly different ($P < 0.05$).

¹ G+ diet was mixed by replacing 1% wheat with same amount of glycine (Redox Pty Ltd., Brisbane, Queensland, Australia).

scores measured in the duodenum ($P < 0.05$) and ileum ($P < 0.05$), showing that birds challenged with Cp without *Eimeria* had a higher lesion score compared to birds that were not challenged with Cp. A Cp × glycine interaction ($P < 0.05$) on lesions in the ileum revealed that birds challenged with Cp but not fed glycine had significantly higher lesion scores compared to those not challenged with Cp.

4. Discussion

The ban of in-feed antibiotics in the European Union in the past decade has resulted in increased interest in developing alternative systems for combatting the effects of NE in the poultry industry (McDevitt et al., 2006). Research into subclinical NE in poultry is hindered by a lack of consistency with NE challenge models between and within different institutes. The species and attenuation of *Eimeria* used in such experiments influences variation in mortality and performance observed. This suggests that inclusion of uniform chemical such as glycine to induce NE could result in a more reproducible NE challenge model. This study demonstrated the roles of glycine supplementation in establishing a subclinical NE challenge model. High levels of protein in feed have been suggested to be favorable for Cp to proliferate (Williams et al., 2001; Lan et al., 2005). Drew et al. (2004) found that broiler chickens fed on 400 g/kg fishmeal as the only protein source in diets had higher Cp counts in ileum and cecum, compared to the birds fed on 400 g/kg soy protein concentrate diets. The amino acid levels of glycine (29.6 g/kg) and methionine (8.4 g/kg) in 400 g/kg fishmeal

diet were elevated (1.3 fold greater) whereas other amino acids decreased compared to 400 g/kg soy protein concentrate diet (glycine: 17.9 g/kg, methionine: 6.2 g/kg). Dahiya et al. (2005) showed that dietary feeding 30.4 g/kg of glycine resulted in significantly higher Cp concentrations in ileum and cecum of broiler chickens than the diets with a series of dietary glycine concentrations (7.5, 15.8 and 42.1 g/kg). This study showed that glycine supplementation did not manifest its effect on NE by negatively affecting bird performance, which is in contrast to the findings of Dahiya et al. (2007). Despite the supplemental glycine level used in this study was relatively low, one possible reason for this opposing observation may be that Dahiya et al. (2007) challenged the birds with Cp starting from d 14 to 21 that resulted in a clinical form of NE with a higher prevalence and severity of intestinal lesions than were observed in the current study. Another possible explanation may lie with differences between the dietary treatments, particularly as the diets were formulated based on different nutrient specifications. This suggests that the ability of glycine supplementation to influence performance in a NE challenge model is dependent on the system used to challenge with Cp and the diets fed to the birds. This implies that different designs may be needed to achieve different severities of disease and highlights that further investigation into NE challenge models is required. Indeed, diet is a major pre-disposing factor for NE, together with the type of Cp and *Eimeria* co-infection (Kaldhusdal and Hofshagen, 1992).

Glycine supplementation promoted the development of gut lesions in this study, which supports the work of Wilkie et al. (2005)

Table 2Effect of *Clostridium perfringens* (Cp) challenge, *Eimeria* spp. and dietary glycine on duodenal, jejunal and ileal necrotic enteritis lesion scores on d 16.

Treatment means	Duodenum	Jejunum	Ileum
Cp × <i>Eimeria</i> × Glycine			
C– E– G–	0.33	0.00 ^b	0.08
C– E– G+	0.33	0.08 ^{ab}	0.00
C– E+ G–	0.50	0.08 ^{ab}	0.00
C– E+ G+	0.25	0.00 ^b	0.08
C+ E– G–	0.67	0.33 ^{ab}	0.42
C+ E– G+	1.00	0.58 ^a	0.33
C+ E+ G–	0.58	0.25 ^{ab}	0.33
C+ E+ G+	0.50	0.25 ^{ab}	0.17
SEM	0.06	0.05	0.04
Cp × <i>Eimeria</i>			
C– E–	0.33 ^b	0.04 ^b	0.04 ^b
C– E+	0.38 ^b	0.04 ^b	0.04 ^b
C+ E–	0.83 ^a	0.46 ^a	0.38 ^a
C+ E+	0.54 ^{ab}	0.25 ^{ab}	0.25 ^{ab}
Cp × Glycine			
C– G–	0.42	0.04 ^b	0.04 ^b
C– G+	0.29	0.04 ^b	0.04 ^b
C+ G–	0.63	0.29 ^{ab}	0.38 ^a
C+ G+	0.75	0.42 ^a	0.25 ^{ab}
<i>Eimeria</i> × Glycine			
E– G–	0.50	0.17	0.25
E– G+	0.67	0.33	0.17
E+ G–	0.54	0.17	0.17
E+ G+	0.38	0.13	0.13
Main effects			
<i>C. perfringens</i>			
Cp–	0.35 ^b	0.04 ^b	0.04 ^b
Cp+	0.69 ^a	0.35 ^a	0.31 ^a
<i>Eimeria</i>			
E–	0.58	0.25	0.21
E+	0.46	0.15	0.15
Glycine ¹			
G–	0.52	0.17	0.21
G+	0.52	0.23	0.15
<i>P</i> -value			
Cp	0.012	0.001	0.001
<i>Eimeria</i>	NS	NS	NS
Glycine	NS	NS	NS
Cp × <i>Eimeria</i>	0.041	0.001	0.016
Cp × Glycine	NS	0.007	0.011
Cp × <i>Eimeria</i> × Glycine	NS	0.019	NS

NS = not significant.

^{a, b}Means not sharing the same superscripts are significantly different ($P < 0.05$).¹ G+ diet was mixed by replacing 1% wheat with same amount of glycine (Redox Pty Ltd., Brisbane, Queensland, Australia).

who found that dietary glycine level positively correlated with Cp numbers in the gut. In this study, there was no significant difference in gastrointestinal lesion scores between the birds challenged with a combination of Cp and glycine or Cp and *Eimeria*, suggesting glycine can potentially be used as a replacement for *Eimeria*. Gut microflora dictates intestinal immunity, suggesting the observed gut lesions and inflammation were likely a result of heightened growth of pathogenic bacteria (Round and Mazmanian, 2009). It is noteworthy that feed intake was reduced by supplying additional glycine in the diet from d 9 to 24, regardless of Cp or *Eimeria* challenge. This coincided with observations by Cave (1983) in which glycine was used to decrease the voluntary feed intake in broiler breeders. However, Corzo et al. (2004) found that dietary glycine improved feed efficiency without affecting average daily gain, which supports the findings of the current study. Enteric infections can impair digestive functionality, meaning NE-challenged birds likely experience malabsorption of nutrients, including protein (Hoerr, 1998). Surprisingly, glycine had no effect on chicken body weight on d 24, but birds that had been fed the grower diet with glycine supplementation were significantly heavier on d 35, despite the lack of supplemental glycine in the finisher diets from

d 24 to 35. Glycine is metabolically important as the uric acid pathway in poultry is glycine-dependent making glycine required for removal of excess nitrogen. Poultry often synthesize insufficient glycine to meet metabolic requirements, highlighting the potential benefits of increasing dietary glycine level (Baker, 2009). Glycine has been shown to enhance the utilization of amino acids and alleviate physiological stress caused by feeding diets with imbalanced amino acid content, known as one of the conditional essential amino acids (Snetsinger and Scott, 1961). There is some evidence suggesting that glycine, together with serine, interact with threonine as well as sulfur amino acids in a way important for birds given low-protein diets (Dean et al., 2006; Ospina-Rojas et al., 2012; Siegert et al., 2015). In the current study, increasing dietary glycine had a positive effect on weight gain at d 35 whereas it also led to a higher lesion scores in the jejunum.

The combination with Cp with *Eimeria* induced a subclinical NE in the current study, as illustrated by reduced weight gain on d 24 without impacting FCR. This is in agreement with the findings of Wu et al. (2010) who used a similar model and successfully produced subclinical NE. In the present study, however, the presence of *Eimeria* did not have any effect on intestinal lesions, indicating that there may be factors other than gut morphological damage that caused performance loss as a result of subclinical NE challenge; for example, loss from triggering the immune system after the infection (Lochmiller and Deerenberg, 2000). This requires further investigation.

5. Conclusions

In conclusion, the current findings show that supplemental dietary glycine used during the grower period resulted in a better growth performance of broilers on d 35. It also made jejunal lesions more severe in birds challenged with Cp, but not with *Eimeria*.

Conflict of interest

The authors declare that they have no conflict of interest.

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