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*Research Note—*

# Relative Disease Susceptibility and Clostridial Toxin Antibody Responses in Three Commercial Broiler Lines Coinfected with *Clostridium perfringens* and *Eimeria maxima* Using an Experimental Model of Necrotic Enteritis

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SUMMARY. Necrotic enteritis is an enteric disease of poultry resulting from infection by *Clostridium perfringens* with coinfection by *Eimeria* spp. constituting a major risk factor for disease pathogenesis. This study compared three commercial broiler chicken lines using an experimental model of necrotic enteritis. Day-old male Cobb, Ross, and Hubbard broilers were orally infected with viable *C. perfringens* and *E. maxima* and fed a high-protein diet to promote the development of experimental disease. Body weight loss, intestinal lesions, and serum antibody levels against α-toxin and necrotic enteritis B-like (NetB) toxin were measured as parameters of disease susceptibility and host immune response. Cobb chickens exhibited increased body weight loss compared with Ross and Hubbard breeds and greater gut lesion severity compared with Ross chickens. NetB antibody levels were greater in Cobb chickens compared with the Ross or Hubbard groups. These results suggest that Cobb chickens may be more susceptible to necrotic enteritis in the field compared with the Ross and Hubbard lines.

RESUMEN. *Nota de Investigación*—Susceptibilidad relativa a la enfermedad y respuesta de anticuerpos contra toxinas clostridiales en tres líneas de pollo de engorde comerciales coinfectadas por *Clostridium perfringens* y *Eimeria maxima* utilizando un modelo experimental de enteritis necrótica.

La enteritis necrótica es una enfermedad entérica de las aves comerciales que resulta de la coinfección por *Clostridium perfringens* y *Eimeria* spp. que constituye un factor de riesgo importante para la patogénesis de la enfermedad. En este estudio se compararon tres líneas de pollos de engorde comerciales utilizando un modelo experimental de enteritis necrótica. Pollos de engorde de un día de edad, de las líneas Cobb, Ross y Hubbard fueron infectados oralmente con *C. perfringens* y con *E. maxima* viables y fueron alimentados con una dieta alta en proteínas para favorecer el desarrollo de la enfermedad experimental. Se midieron la pérdida de peso corporal, las lesiones intestinales, y los niveles séricos de anticuerpos contra la toxina α y contra la toxina de enteritis necrótica similar a la toxina B (NetB) y se analizaron como parámetros de susceptibilidad a la enfermedad, también se midió la respuesta inmune del huésped. Los pollos de la línea Cobb mostraron una mayor pérdida de peso corporal en comparación con los pollos de las líneas Ross y Hubbard y mayor severidad de lesiones intestinales en comparación con los pollos Ross. Los niveles de anticuerpos contra la toxina NetB fueron mayores en pollos Cobb en comparación con los pollos de las líneas Ross y Hubbard. Estos resultados sugieren que los pollos Cobb pueden ser más susceptibles a la enteritis necrótica en el campo en comparación con las líneas Hubbard y Ross.

Key words: coccidia, protozoa, intestine, toxin

Abbreviations: Ig = immunoglobulin; MHC = major histocompatibility complex; NetB = necrotic enteritis B-like; $OD_{450}$ = optical density at 450 nm; PBS = phosphate-buffered saline; PBS-T = phosphate-buffered saline containing 0.05% Tween 20

Necrotic enteritis is a major enteric disease of poultry caused by infection with *Clostridium perfringens* (4,29,30). Necrotic enteritis is widespread in broilers, imposing a significant economic burden on the poultry industry worldwide. Necrotic enteritis has primarily been controlled with antibiotics. One of the negative consequences associated with the prohibition of antibiotic growth promoters in commercial poultry production is the increase in intestinal infectious diseases, such as necrotic enteritis, coccidiosis, and cryptosporidiosis (2). Identification of antibiotic-free, alternative disease control strategies has been hindered by the difficulty of experimentally reproducing necrotic enteritis by *C. perfringens* infection alone (3,19). Predisposition to experimental disease has been achieved by a high-protein diet and intestinal damage after coinfection with the apicomplexan protozoa *Eimeria* spp. (13,31).

Several publications have described the relationship between performance traits of commercial broiler breeds and their ability to mount immunologic responses (11,22). However, no information is available on the susceptibility of different chicken lines to experimental necrotic enteritis. Therefore, the current study was performed to compare disease susceptibility and host antibody responses to *C. perfringens* toxins in three commercial broiler chicken lines using an experimental model of necrotic enteritis.

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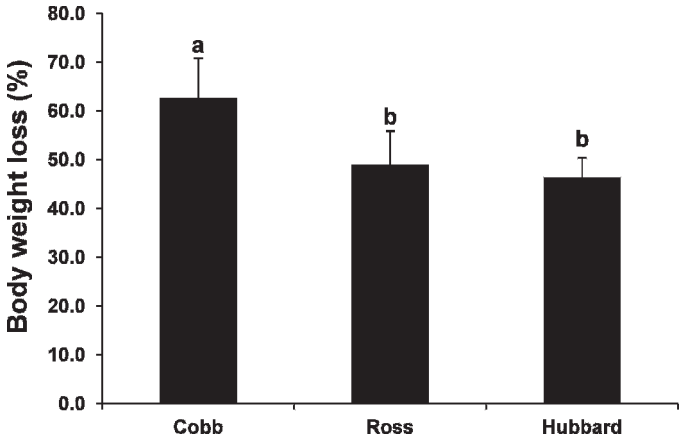


Fig. 1. Body weight loss in commercial chicken breeds during experimental necrotic enteritis. Chickens were uninfected or coinfected with *E. maxima* on day 14 and *C. perfringens* on day 18 posthatch. Body weights were measured between days 14 and 20. Each bar represents the mean ± SD ($n = 15$). Bars not sharing the same letters are significantly different according to the Duncan's multiple range test ($P < 0.05$).

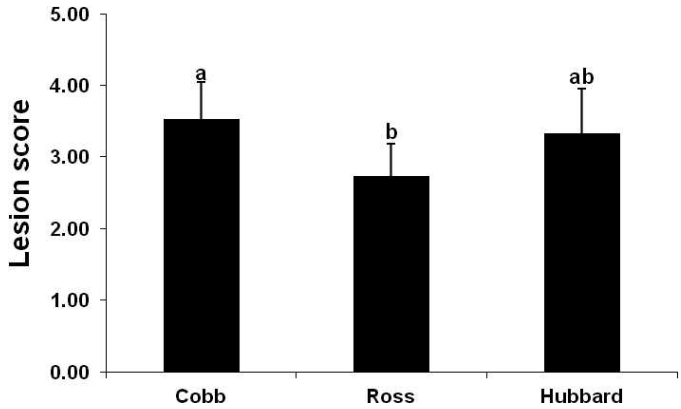


Fig. 2. Intestinal lesion in commercial chicken breeds during experimental necrotic enteritis. Chickens were uninfected or coinfected with *E. maxima* on day 14 and *C. perfringens* on day 18 posthatch. Intestinal lesion scores were made on day 20 using a scale from 0 (none) to 4 (high). Each bar represents the mean ± SD of the combined observations on five chickens by three independent observers ($n = 15$). Bars not sharing the same letters are significantly different according to the Duncan's multiple range test ($P < 0.05$).

## MATERIALS AND METHODS

**Chickens.** One hundred and fifty 1-day-old male Cobb 700, Ross 708, and Hubbard chickens were obtained from Mountaire Farms (Millsboro, DE), housed in the Petersime starter brooder units, and provided with feed and water *ad libitum* (9). Chickens were kept in brooder pens in a disease-free facility for 14 days posthatch and transferred into large hanging cages (two birds/cage) at a separate location where they were infected and kept until the end of the experimental period.

**Challenge infections.** Experimental necrotic enteritis was produced by coinfection with *Eimeria maxima* and *C. perfringens* as described previously (9,14,19). Chickens were randomly divided into two groups—uninfected control and coinfected—of 25 birds of each breed. Both infections were via oral gavage with *E. maxima* ($1.0 \times 10^4$ oocysts/bird) given on day 14 posthatch and *C. perfringens* strain Del-1 ($1.0 \times 10^9$ colony-forming units/bird) given on day 18. Uninfected controls received phosphate-buffered saline (PBS). Birds were fed an antibiotic-free, certified organic starter diet containing 17% crude protein between days 1 and 14, followed by a standard grower diet containing 24% crude protein between days 14 and 28. All animal protocols were approved by the Beltsville Area Institutional Animal Care and Use Committee.

**Body weights.** Body weights of all chickens were measured between days 14 and 20 posthatch (days 0–6 postinfection with *E. maxima*) as described previously (9).

**Gut lesion scores.** On day 20 posthatch, five chickens from each group were randomly selected, intestines were removed, and necrotic enteritis lesions were scored on a numerical scale from 0 (none) to 4 (high) as described previously (23). Lesion scores were evaluated in a blinded manner by three independent observers.

***C. perfringens* α-toxin and necrotic enteritis B-like (NetB) toxin serum antibody levels.** On day 20 posthatch, five chickens from each group were randomly selected, blood was collected by cardiac puncture immediately after euthanatization, and sera were obtained by low-speed centrifugation. Sera from individual chickesn in each group were pooled and used in an ELISA to measure α-toxin and NetB toxin antibody levels as described previously (16). In brief, 96-well microtiter plates were coated overnight with 1.0 μg/well purified recombinant α-toxin or NetB proteins (15). The plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with PBS containing 1% bovine serum albumin. Sera (100 μl/well) were incubated for 2 hr at room temperature with gentle agitation, the plates were washed with PBS-T, and bound antibody was detected with peroxidase-conjugated rabbit anti-chicken IgG (Sigma, St. Louis, MO) and peroxidase-specific substrate. Optical density at 450 nm ($OD_{450}$) was measured with an automated microplate reader (Bio-Rad Laboratories, Hercules, CA). Antibody titers were defined as the highest serum dilution producing $OD_{450} \geq 0.4$ and were expressed as log 10.

**Statistical analysis.** All data were subjected to one-way analysis of variance using SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL). Mean values of treatment groups were compared using the Duncan's multiple range test, and differences were considered statistically significant at $P < 0.05$.

## RESULTS

**Growth performance.** Cobb chickens coinfected with *E. maxima* and *C. perfringens* exhibited significantly increased body weight loss between days 0 and 6 postinfection with *E. maxima* compared with the Ross or Hubbard breeds (Fig. 1).

**Gut lesions.** Cobb chickens coinfected with *E. maxima* and *C. perfringens* exhibited significantly increased intestinal lesion scores at day 2 postinfection with *C. perfringens* compared with Ross chickens (Fig. 2). No differences in lesion scores were seen when comparing Cobb *vs*. Hubbard or Ross *vs*. Hubbard.

**Serum antibody responses.** All chickens coinfected with *E. maxima* and *C. perfringens* had significantly greater levels of α-toxin and NetB toxin serum IgG antibodies at day 2 postinfection with *C. perfringens* compared with uninfected controls (Fig. 3). No differences in α-toxin antibody levels were seen among the coinfected Cobb, Ross, and Hubbard lines (Fig. 3A). NetB antibody levels were greater in coinfected Cobb chickens compared with the Ross and Hubbard strains (Fig. 3B).

## DISCUSSION

Necrotic enteritis is an emerging and devastating poultry disease caused by *C. perfringens* infection, typically following intestinal damage by coccidiosis. Prior studies have demonstrated that experimental *C. perfringens* infection alone produces some of the clinical signs associated with necrotic enteritis on commercial poultry farms (17). Chalmers *et al.* (1) reported that only one of five *C. perfringens* isolates taken from field cases of necrotic enteritis, combined with a high-protein diet, produced greater weight loss,

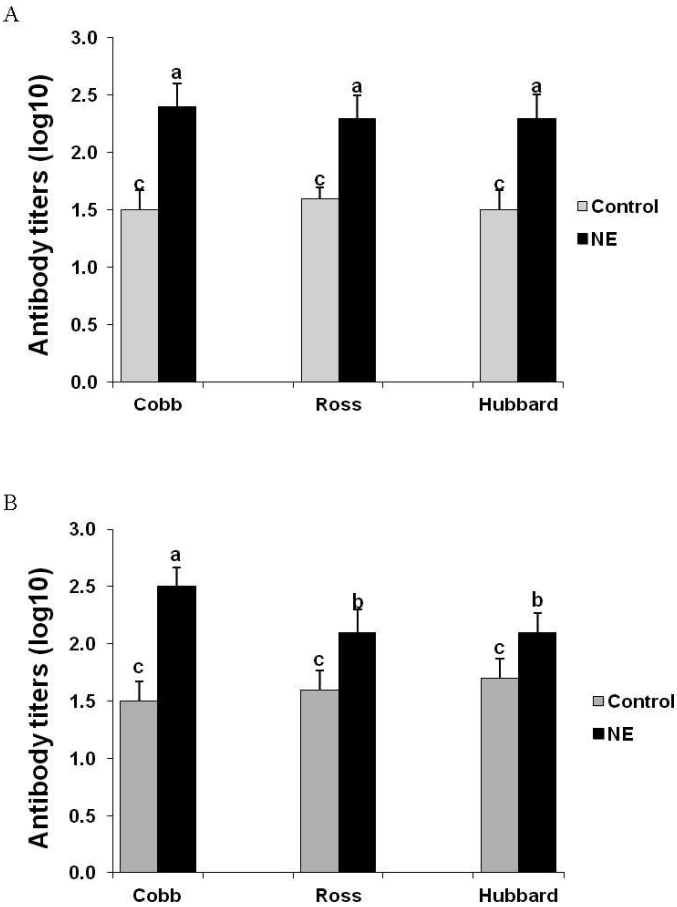


Fig. 3. Antibody responses to α-toxin and necrotic enteritis B-like (NetB) toxin in commercial chicken breeds during experimental necrotic enteritis. Chickens were uninfected (control) or coinfected with *E. maxima* on day 14 and *C. perfringens* on day 18 posthatch (NE). Serum immunoglobulin G antibody levels against α-toxin (A) and NetB toxin (B) were measured by ELISA on day 20. Each bar represents the mean ± SD ($n = 5$). Bars not sharing the same letters within each panel are significantly different according to the Duncan's multiple range test ($P < 0.05$).

increased gut lesions, and higher mortalities compared with the other four bacterial strains and uninfected controls. The *Eimeria-C. perfringens* coinfection model system that we (8,9,13,19) and others (3,6,28,31,32) have described more efficiently replicates the disease seen in commercial flocks. Specifically, although *E. maxima* infection alone does not produce intestinal lesions typical of necrotic enteritis, and *C. perfringens* infection alone induces pathologic lesions of mild severity, both microorganisms together act synergistically to produce high-grade, severe gut lesions that are characteristic of disease in the field (13).

Commercial poultry breeding has amongst its many objectives the improvement of production potential and increase in disease resistance. Often, however, selection of high-growth-rate broilers has led to decreased disease resistance (7,20). Blunted humoral and cellular immune responses have been described in modern broiler strains selected for high growth performance. Miller *et al.* (18) reported a negative correlation between body weight and antibody levels to sheep red blood cells as an experimental marker of the humoral response. Qureshi and Miller (24) observed that commercial broiler lines differed in several baseline functional parameters of their mononuclear phagocytes. Given that host immunity plays a protective role, albeit incomplete, in the development and progression of avian necrotic enteritis (13), it is not unreasonable to predict that poultry genetics also plays a major role in this disease. Until now, however, limited information has been available on the effect of chicken breeds on necrotic enteritis susceptibility. Siegel *et al.* (27) described a natural outbreak of necrotic enteritis and its association with the major histocompatibility complex (MHC) in laying hens.

Antibody production against a variety of antigens is linked to the chicken MHC (12), and genetic control of the humoral immune response in egg-type chickens has been documented (5,21,26). Higher α-toxin and NetB toxin serum IgG antibodies were detected in the three chicken breeds afflicted with experimental necrotic enteritis compared with uninfected controls. Although the role of humoral immunity in protection against necrotic enteritis remains to be determined, Ritter (25) proposed that early exposure to *C. perfringens* may induce an antibody response to clostridial toxins that prevents later infections. Indeed, animals vaccinated with *C. perfringens* toxoids were protected against lethal challenge infections, and recombinant α-toxin vaccines induced protective antibody responses (10). We also observed higher levels of serum antibodies against NetB toxin in Cobb chickens compared with the Ross and Hubbard lines. These results suggest that genetic differences between some commercial broiler chicken lines may be responsible for restricted (i.e., NetB) anticlostridial toxin antibody responses. However, because Cobb chickens were most susceptible to experimental necrotic enteritis, neither α-toxin nor NetB toxin IgG antibodies appear to confer protection in our disease model. Other factors are more likely to regulate host resistance against experimental disease, such as the route and dose of infection and the local IgA antibody response in the gut.

In conclusion, Cobb broiler chickens had greater body weight loss, increased gut lesion scores, and increased NetB serum IgG antibody levels after experimental coinfection with *E. maxima* and *C. perfringens* compared with Ross or Hubbard chicken lines. Additional studies are necessary to better understand the interactions between host and pathogen, as well as between pathogen and pathogen (*C. perfringens-E. maxima*) during avian necrotic enteritis.

## REFERENCES

1. Chalmers, G., H. L. Bruce, D. L. Toole, D. A. Barnum, and P. Boerlin. Necrotic enteritis potential in a model system using *Clostridium perfringens* isolated from field outbreaks. Avian Dis. 51:834–839. 2007.

2. Collett, S. Recent developments in drug free programmes for poultry. In: Recent developments in animal feeds and feeding. Proc. AFMA Forum, Sun City, South Africa. March 10–12. 2004.

3. Collier, C. T., C. L. Hofacre, A. M. Payne, D. B. Anderson, P. Kaiser, R. I. Mackie, and H. R. Gaskins. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. Vet. Immunol. Immunopathol. 122:104–115. 2008.

4. Cooper, K. K., and J. G. Songer. Necrotic enteritis in chickens: a paradigm of enteric infection by *Clostridium perfringens* type A. Anaerobe 15:55–60. 2009.

5. Dunnington, E. A., W. E. Briles, R. W. Briles, and P. B. Siegel. Immunoresponsiveness in chickens: association of antibody production and the B system of the major histocompatibility complex. Poult. Sci. 75:1156–1160. 1996.

6. Forder, R. E., G. S. Nattrass, M. S. Geier, R. J. Hughes, and P. I. Hynd. Quantitative analyses of genes associated with mucin synthesis of broiler chickens with induced necrotic enteritis. Poult. Sci. 91:1335–1341. 2012.

7. Han, P. F., and J. R. Smyth Jr. The influence of growth rate on the development of Marek's disease in chickens. Poult. Sci. 51:975–985. 1972.

8. Hong, Y. H., W. Song, S. H. Lee, and H. S. Lillehoj. Differential gene expression profiles of β-defensins in the crop, intestine, and spleen using a necrotic enteritis model in 2 commercial broiler chicken lines. Poult. Sci. 91:1081–1088. 2012.

9. Jang, S. I., H. S. Lillehoj, S. H. Lee, K. W. Lee, E. P. Lillehoj, Y. H. Hong, D. J. An, W. S. Jeong, J. E. Chun, F. Bertrand, L. Dupuis, and S. Deville. Vaccination with *Clostridium perfringens* recombinant proteins in combination with Montanide™ ISA 71 VG adjuvant increase protection against experimental necrotic enteritis in commercial broiler chickens. Vaccine 30:5401–5406. 2012.

10. Johnson, S. Antibody responses to clostridial infection in humans. Clin. Infect. Dis. 25:S173–S177. 1997.

11. Kreukniet, M. B., N. Gianotten, M. B. Nieuwland, and H. K. Parmentier. *In vitro* T cell activity in two chicken lines divergently selected for antibody response to sheep erythrocytes. Poult. Sci. 73:336–340. 1994.

12. Lamont, S. J. Impact of genetics on disease resistance. Poult. Sci. 77:1111–1118. 1998.

13. Lee, K. W., H. S. Lillehoj, W. Jeong, H. Y. Jeoung, and D. J. An. Avian necrotic enteritis: experimental models, host immunity, pathogenesis, risk factors, and vaccine development. Poult. Sci. 90:1381–1390. 2011A.

14. Lee, K. W., H. S. Lillehoj, G. Li, M. S. Park, S. I. Jang, W. S. Jeong, H. Y. Jeoung, D. J. An, and E. P. Lillehoj. Identification and cloning of two immunogenic *Clostridium perfringens* proteins, elongation factor Tu (EF-Tu) and pyruvate:ferredoxin oxidoreductase (PFO) of *C. perfringens.* Res. Vet. Sci. 91:e80–e86. 2011.

15. Lee, K. W., H. S. Lillehoj, M. S. Park, S. I. Jang, G. D. Ritter, Y. H. Hong, W. Jeong, H. Y. Jeoung, D. J. An, and E. P. Lillehoj. *Clostridium perfringens* α-toxin and NetB toxin antibodies and their possible role in protection against necrotic enteritis and gangrenous dermatitis in broiler chickens. Avian Dis. 56:230–233. 2012.

16. Li, G., H. S. Lillehoj, K. W. Lee, S. I. Jang, P. Marc, G. Cyril, D. Ritter, D. A. Bautista, K. Phillips, A. P. Neumann, T. G. Rehberger, and G. R. Siragusa. An outbreak of gangrenous dermatitis in commercial broiler chickens. Avian Pathol. 39:247–253. 2010.

17. Mikkelsen, L. L., J. K. Vidanarachchi, C. G. Olnood, Y. M. Bao, P. H. Selle, and M. Choct. Effect of potassium diformate on growth performance and gut microbiota in broiler chickens challenged with necrotic enteritis. Br. Poult. Sci. 50:66–75. 2009.

18. Miller, L. L., P. B. Siegel, and E. A. Dunnington. Inheritance of antibody response to sheep erythrocytes in lines of chickens divergently selected for fifty-six-day body weight and their crosses. Poult. Sci. 71:47–52. 1992.

19. Park, S. S., H. S. Lillehoj, P. C. Allen, D. W. Park, S. FitzCoy, D. A. Bautista, and E. P. Lillehoj. Immunopathology and cytokine responses in broiler chickens coinfected with *Eimeria maxima* and *Clostridium perfringens* with the use of an animal model of necrotic enteritis. Avian Dis. 52:14–22. 2008.

20. Parmentier, H. K., Nieuwland, M. G., Rijke, E., G. De Vries Reilingh, and J. W. Schrama. Divergent antibody responses to vaccines and divergent body weights of chicken lines selected for high and low humoral responsiveness to sheep red blood cells. Avian Dis. 40:634–44. 1996.

21. Pinard, M. H., J. A. M. van Arendonk, M. G. B. Nieuwland, and A. J. van der Zijpp. Divergent selection for humoral immune responsiveness in chickens: distribution and effects of major histocompatibility complex types. Genet. Sel. Evol. 25:191–203. 1993.

22. Praharaj, N. K., E. A. Dunnington, and P. B. Siegel. Growth, immunoresponsiveness, and disease resistance of diverse stocks of chickens reared under two nutritional regimes. Poult. Sci. 74:1721–1729. 1995.

23. Prescott, J. F. The prevention of experimentally induced necrotic enteritis in chickens by avoparcin. Avian Dis. 23:1072–1074. 1979.

24. Qureshi, M. A., and L. Miller. Comparison of macrophage function in several commercial broiler genetic lines. Poult. Sci. 70:2094–2101. 1991.

25. Ritter, G. Proposed pathogenesis of gangrenous dermatitis in chickens and attempts at experimental reproduction. In: Proc. 41st National Meeting on Poultry Health and Processing, Ocean City, MD. pp. 59–66. 2006.

26. Siegel, P. B., and W. B. Gross. Production and persistence of antibodies in chickens to sheep erythrocytes. 1. Directional selection. Poult. Sci. 59:1–5. 1980.

27. Siegel, P. B., A. S. Larsen, C. T. Larsen, and E. A. Dunnington. Resistance of chickens to an outbreak of necrotic enteritis as influenced by major histocompatibility genotype and background genome. Poult. Sci. 72:1189–1191. 1993.

28. Timbermont, L., A. Lanckriet, A. R. Gholamiandehkordi, F. Pasmans, A. Martel, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. Origin of *Clostridium perfringens* isolates determines the ability to induce necrotic enteritis in broilers. Comp. Immunol. Microbiol. 32:503–512. 2009.

29. Timbermont, L., F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathol. 40:341–347. 2011.

30. Van Immerseel, F. V., J. De Buck, F. Pasmans, G. Huyghebaert, F. Hasesebrouck, and R. Ducatelle. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian Pathol. 33:537–549. 2004.

31. Williams, R. B., R. N. Marshall, R. M. La Ragione, and J. Catchpole. A new method for the experimental production of necrotic enteritis and its use for studies on the relationships between necrotic enteritis, coccidiosis and anticoccidial vaccination of chickens. Parasitol. Res. 90:19–26. 2003.

32. Wu, S. B., N. Rodgers, and M. Choct. Optimized necrotic enteritis model producing clinical and subclinical infection of *Clostridium perfringens* in broiler chickens. Avian Dis. 54:1058–1065. 2010.

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