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Researcher Contact Details
Shubiao Wu
School of Environmental and Rural Science
The University of New England

Phone : +612 6773 2238
Fax: +612 6773 3922
Email: shubiao.wu@une.edu.au

In submitting this report, the researcher has agreed to the Australian Poultry CRC publishing this material in its edited form.

Australian Poultry CRC Contact Details
PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: info@poultrycrc.com.au
Website: http://www.poultrycrc.com.au

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Executive Summary

In this study, we aimed to assess the role of *Eimeria* infection and fishmeal feeding in the UNE NE challenge model and the capability of real-time PCR quantification of bacterial 16S rDNA as a viable replacement for traditional bacterial culture techniques.

The experiments were designed to test the following hypotheses: 1. *Eimeria* infection and fishmeal feeding are necessary for full NE challenge experiment; and 2. Quantitative real time PCR can improve the accuracy and efficiency of enumeration of bacteria in the intestinal tract of broiler chicken compared to the conventional *in vitro* culture.

The results showed that the combination of *Eimeria* administration and fishmeal feeding had a significant effect on induction of NE disease and mortality of the birds subjected to Cp challenge. High fishmeal feeding alone did not lead to significant mortality of Cp challenged birds, but showed a significantly higher Cp counts than the control based on the real-time PCR assay. *Eimeria* administration had a significant effect on bird mortality caused by NE but did not show an impact on the Cp count compared to the corresponding Cp challenged groups with various levels of fishmeal feeding. In accordance with the time course of bird mortality, it can be determined that at least the third Cp inoculation of the 3 successive oral gavage processes did not contribute to the death of the birds, and therefore can be omitted in the challenge procedure. It can be recommended that 25-50% fishmeal feeding, *Eimeria* (*E. acervulina*, *E. maxima* and *E. tenella*) inoculation, two oral Cp inoculations and appropriate ambient temperatures and diets (stated in methodology) are suitable for an NE challenge experiment as a model.

For Cp enumeration, quantitative real-time PCR proved to be compatible to conventional *in vitro* culture method with higher efficiency and accuracy. The correlation analysis of the means of the treated groups showed that these two methods are highly correlated (R^2=0.845). However, the real-time PCR method produced higher accuracy than the culture method as demonstrated by comparison of the relative standard deviations (RSD). The RSD of culture method were as high as 10.8 times of the real-time PCR method.

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Introduction

Necrotic enteritis (NE) leads to over $2 billion annual loss of the world's broiler industry (Dahiya *et al.*, 2006). The causative agent of the disease is the bacterium *Clostridium perfringens* (Cp) which can be found in litter, faeces and intestinal contents. It has been observed that Cp types A and C causes NE with type A being the main type, and other factors such as coccidiosis (caused by *Eimeria* infection), temperature and diet contribute to the outbreak of this disease.

A NE challenge model involving administration of *Eimeria* followed by Cp challenge has been frequently applied in studies addressing broiler chicken nutrition and health. However, reproducibility of the model is not guaranteed (Kaldhusdal *et al.*, 1999). There have been cases where bird mortality in challenged groups exceeded expectation as demonstrated by our recent study funded by the Poultry CRC (Project 06-18). It was revealed that coccidiosis could lead to high mortality in challenged birds. Hence, a revision of the model to determine the role of *Eimeria* in the challenge process is important to improve the reproducibility, safety and predictability of the NE challenge model. In addition, other factors such as ambient temperature, diet formulation and timing of challenge are also worthy of attention in the model.

Bacteria in the chicken gastrointestinal tract play an important role in health (Lu *et al.*, 2003). Therefore, quantification of the major bacterial communities in the chicken gut is essential for the researcher to monitor changes in microbial ecology in experiments involving Cp challenge. Traditionally, analysis of gastrointestinal communities has depended on bacterial culture and colony counting methods and microscopy. However, the methodologies involved are time consuming and require researchers to possess substantial microbiological expertise. Furthermore, potential bias is present in the results has been identified, since only those bacteria whose physiological and metabolic requirements are reproducible in vitro, are able to be cultivated (Walter *et al.*, 2000).

Molecular approaches have been applied to rapid characterisation of bacteria. These include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), conventional polymerase chain reaction (PCR), and terminal restriction fragment length polymorphism (T-RFLP). These approaches are able to identify relevant bacterial groups but the drawback of these techniques is that they are not fully quantitative, and so cannot act as an alternative method to colony counting following in vitro culture. In contrast, real-time PCR can be used to quantify the bacteria, as the number of target gene copies can be determined from the DNA extracted from samples obtained from challenged broilers. Hence, real-time PCR has recently been used to enumerate bacteria in the gastrointestinal tract (Wise and Siragusa, 2007). However, comparison of the two methods (traditional in vitro culture and real-time PCR) and their correlation has been lacking.

In this study, we assessed the role of *Eimeria* infection and fishmeal feeding in the UNE NE challenge model and the capability of real-time PCR quantification of bacterial 16S rDNA as a viable replacement for traditional bacterial culture techniques. The correlation between the results produced from real-time PCR and colony counting were also investigated.

Objectives

The project investigated the optimal conditions with *Eimeria* infection, and at different levels of fishmeal feeding for the NE challenge in broiler chickens; and assessed quantitative real-time PCR of bacteria DNA as an alternative method to bacterial *in vitro* culture for the enumeration of bacteria in intestinal tract of broiler chicken.

The experiments were designed to test the following hypotheses:

1. *Eimeria* infection and fishmeal feeding are necessary for full NE challenge experiment.
2. Quantitative real-time PCR can improve the accuracy and efficiency of enumeration of bacteria in the intestinal tract of broiler chicken compared to the conventional *in vitro* culture.

Methodology

Birds and diets

1512 day-old Cobb male broiler chickens (Baiada hatchery, Kootingal, NSW, Australia) were used at the start of experiment, and the birds were culled to 25 per cage on d7. 1350 birds were raised for 5 weeks in 54 floor pens in a temperature-controlled room (33^{o}C-34^{o}C during week 1, decreased 3^{o}C each week to 21-23^{o}C by the third week) at Kirby research station at The University of New England. The birds were subjected to artificial fluorescence illumination of 16 h per day. Each cage was assigned to one of 9 treatment groups (Table 1) with six replicates per treatment (25 birds/cage). Throughout this report, NFM-, LFM- and HFM- represent the treatments with no, 25% and 50% fishmeal feeding respectively, but no Cp and *Eimeria* infections; NFM+Cp, LFM+Cp and HFM+Cp the treatments with no, 25% and 50% fishmeal feeding respectively, and Cp challenge, but no *Eimeria* infection; and NFM+, LFM+ and HFM+ the treatments with no, 25% and 50% fishmeal feeding respectively, Cp challenge and *Eimeria* infection.

Table 1 Experimental design of the treatments with fishmeal feeding, *Eimeria* infection, and *C. perfringens* challenge.

Treatment	Fishmeal	*Eimeria*	*C. perfringens*
1 (NFM-)	None	No	None
2 (LFM-)	Low	No	None
3 (HFM-)	High	No	None
4 (NFM+Cp)	None	No	Yes
5 (LFM+Cp)	Low	No	Yes
6 (HFM+Cp)	High	No	Yes
7 (NFM+)	None	Yes	Yes
8 (LFM+)	Low	Yes	Yes
9 (HFM+)	High	Yes	Yes

Nutrient and dietary composition of the starter and finisher diets are shown as in Table 2. The birds were given the starter diet during days 1 to 7, appropriate fishmeal added to induce stress on GIT during days 8 to 14, fishmeal removed on day 14, used starter diet again during days 15 to 21, and fed finisher during days 22 to 35. Birds had *ad lib* access to feed and water throughout the experiment. Live weights of the birds were measured on days 7, 14, 21, 28 and 35 d of the experiment.

Table 2 Composition of experimental diets (in g/kg)

Ingredients	Sarter	Finisher
Wheat	266	289.5
Barley	-	22
Sorghum	400	400
Tallow	10.5	20
Sunflower/soy oil	10	1
Canola meal	25	-
Soybean meal	250	228
Limestone	14.5	15
KYNOFOS / BIOFOS MDCP	10.5	11
Salt	1.5	1.5
Sodium bicarbonate	2.5	2.5
Choline chloride 75%	0.3	0.6
Dl-methionine	2.6	2.4
L-lysine	3.4	3.3
L-threonine	0.6	0.5
RAP STD CHK/BRL FIN PMX	2	2
Phyzyme XP5000 broiler	0.1	0.1
Ronozyme WX CT	0.3	0.3

Necrotic enteritis challenge

Birds were vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease. The research facility was thoroughly cleaned and disinfected prior to bird placement. On day 9, birds in appropriate groups to be challenged were given per os a suspension of 5000 sporulated oocytes of *Eimeria acervulina*, *E. maxima*, *E. tenella* (Bioproperties Pty Ltd., Glenorie, NSW) in 1 mL sterile PBS. Birds in other groups were given sterile PBS in place of *Eimeria*. On days 14, 15 and 16, birds to be challenged were inoculated per os with 1 mL of *C. perfringens* suspension at a concentration of 10^8-10^9 CFU/mL. A primary poultry isolate of *C. perfringens* type A (CSIRO Livestock Industries, Geelong) was incubated overnight at 39^{o}C in 1000 mL of thioglycollate broth containing starch (10 g/L) and casitone (5 g/L) to obtain the challenge inoculum. Birds in unchallenged group received 1 mL of sterile thioglycollate broth.

Sample collection

On day 14 prior to *C. perfringens* inoculation, and on day 17, two birds were randomly chosen in each cage and sacrificed for sample collections. All birds were euthanised by cervical dislocation, total body weight of each bird was recorded, and bursa were removed and weighed on day 17.

The contents of the ileum were pooled separately for the birds sacrificed in each replicate. pH of the contents was measured, and approximately 1 g of the digesta was collected for microbial culture, and the remaining digesta was stored for determination of volatile fatty acid analysis. For each bird, a section of approximately 3 cm ileum (including digesta) was taken at the mid-point between Meckel's diverticulum and caecal tonsils per bird were sampled for quantitative PCR analysis.

Short chain fatty acids

For measurement of short chain fatty acid (volatile fatty acids, lactic and succinic acids) concentrations, about 2.0 g of thawed gizzard, ileal and caecal sample was suspended in 1.0 mL of 0.01M 2-ethylbutyric acid as internal standard and thoroughly mixed, followed by centrifugation at 25,000 x g at 5°C for 20 min. To a sample of 1 mL supernatant, 0.5 mL of concentrated HC1 (36%) and 2 mL of diethyl ether were added

and thoroughly mixed, followed by centrifugation at 2000 x g at 5°C for 15 min. Four hundred μL of supernatant (the ether phase) were mixed with 40 μL N-methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA), and incubated at 80°C for 20 min. The mixture was then left at room temperature for at least 48 hrs before concentration quantification of the organic acids. The concentration of organic acids was quantified using a Varian CP3800 gas chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA). Varian Star 5.52 chromatography workstation (integration system) software (Varian Analytical Instruments, Palo Alto, CA, USA) was used for data processing. Total organic acid concentration is the sum of the all organic acids observed in a sample, expressed as μmol/g digesta.

Enumeration of ileal bacteria

Intestinal digesta samples in pre-reduced salt medium were homogenised for 2 min in CO_2-flushed plastic bags using a MiniMix® bag mixer (Interscience, St. Nora, France) and serial diluted in 10-fold increments according to the technique of Miller and Wolin (1974). An aliquot (100 μL) was plated on the following media. Total anaerobic bacteria were enumerated on Wilkins-Chalgren anaerobic agar (Oxoid, CM0619) after anaerobic incubation at 39°C for 7 days. Coliform bacteria and lactose-negative enterobacteria were counted on MacConkey agar (Oxoid, CM0115) after aerobic incubation at 39°C for 24 h. The population of Cp were counted on Tryptose-Sulfite-Cycloserine and Shahidi-Ferguson Perfringens agar base (TSC & SFP) (Oxoid, CM0587) mixed with egg yolk emulsion (Oxoid, SR0047) and Perfringens (TSC) selective supplement (Oxoid, SR0088E) according to the pour-plate technique, where plates were overlaid with the same agar after spreading the inoculums. *C. perfringens* plates were incubated anaerobically for 48 h at 39°C prior to counting. Bacterial numbers were expressed as $\log_{10}$ CFU/g digesta.

DNA extraction and quantitative PCR

Extraction of DNA from ileal content was conducted using a QIAamp DNA Stool kit (QIAgen, Hilden, Germany) following the instruction of manufacture with slight modifications. 180–220 mg frozen digesta was taken from stored samples and 300 mg glass beads (0.1mm, Biospec Products, Bartlesville, OK, USA) used to break the cells in 400 μL Buffer ASL by shaking the sample on a Mini Beadbeater (Biospec Products, Bartlesville, OK, USA) for 30 sec. The cells were then lysed after adding 1 mL of ASL by heating the suspension for 5 min at 95°C. Stool particles were pelleted by centrifugation of the lyses and the supernatant was treated by the InhibitEX tablet to absorb the PCR inhibitors. The InhibitEX matrix bound with the inhibitors was pelleted by centrifugation and 200 μL of supernatant was digested in buffer AL at 70°C for 10 min by proteinase K to remove the proteins bound. DNA was precipitated by adding 200 μL of ethanol and captured on the QIAamp spin column. The DNA was washed by 500 μL buffers AW1 and AW2, and eluted by 50 μL TE buffer (10 mM Tris, 1 mM EDTA, pH=8). DNA was considered as good quality when the values of $A_{260/280}$ and $A_{260/230}$ were > 1.8 respectively.

Quantitative real-time PCR assay followed the methods of Wise and Siragusa (2005). Taqman Universal PCR Master Mix (Applied Biosystems) was employed for the real-time PCR with a primer pair (CPerf165F, 5'-CGCATAACGTTGAAAGATGG-3'; and CPerf269R, 5'-CCTTGGTAGGCCGTTACCC-3') specific to Cp 16 S rDNA. The PCR was performed on a Rotorgene 6500 real-time PCR machine (Corbett, Sydney, Australia) with 3 replicates for each sample. A C_t average from the replicates were used for data analysis. The threshold was automatically selected by the software accompanied with Rotorgene 6500. Serial dilutions of a *C. perfringens* DNA with known concentration were used to construct standard curve. The corresponding number of cells was calculated according the genome size of *C. perfringens*. Bacterial numbers were expressed as $\log_{10}$ (genomic DNA copy number)/g digesta.

Statistical analyses

All data were analysed using the statistical package Minitab® for Windows 12.1 (Minitab Inc., State College, USA). An analysis of variance was performed using the one way ANOVA procedure and significant differences among the treatment groups were determined by Tukey Multiple Comparison test of means. As mortality, volatile fatty acids, bacterial enumeration and quantitative PCR data were not normally distributed, these were analysed by the non-parametric Kruskal-Wallis test. To assess the correlation between real-time PCR method and the culture method, simple linear regression of the treatment means produced by these two methods was performed using the Minitab® for Windows 12.1 (Minitab Inc., State College, USA), and relative standard deviations of real-time PCR and culture methods were calculated and compared .

Results

Bird growth

Bird body weight did not show significant difference between the treatments until d28. On d28 and d35, the HFM+ birds showed a significant decrease in body weight compared to the NFM- and LFM- birds ($P < 0.05$) (Table 3).

Mortality

During the week following the challenge of Cp (d15-21), the mortality of the HFM+ and LFM+ birds increased significantly compared to the NFM- birds (Table 4). However, the mortality of NFM+ did not differ significantly from the birds in control groups (NFM-, LFM- or HFM-). Significant differences were not observed during the weeks 4 and 5. According to necropsy examinations, the death of the birds during days 15-21 was caused mainly by NE (33/36 or 92%). The mortality of the birds during d15-21 occurred largely during the first 3 days following the first Cp infection (31/36 or 86%), and therefore at least third inoculation of Cp did not contribute to the death of the birds caused by NE.

Table 3 Body weights of birds with different treatments [1].

Treatment	d7	d14	d21	d28	d35
1 (NFM-)	133.8±3.2	319.5±7.9	664.7±12.0	1298.2±14.5b	1995.7±19.6b
2 (LFM-)	130.4±3.9	324.0±13.8	693.3±41.1	1299.5±40.8b	2000.8±42.0b
3 (HFM-)	132.0±3.1	327.1±4.9	682.9±35.4	1267.1±32.7ab	1979.4±42.1ab
4 (NFM+Cp)	124.6±3.0	311.6±9.6	659.8±16.2	1248.8±27.0ab	1933.0±28.4ab
5 (LFM+Cp)	135.5±2.5	309.4±8.5	705.9±37.7	1287.6±25.9ab	1989.4±38.9ab
6 (HFM+Cp)	127.4±2.3	313.4±7.4	669.4±48.9	1249.6±21.4ab	1936.0±31.7ab
7 (NFM+)	131.4±2.9	328.5±14.3	659.2±8.4	1243.3±16.5ab	1938.3±25.2ab
8 (LFM+)	132.5±3.3	313.0±8.4	647.0±23.1	1238.8±28.6ab	1929.2±32.5ab
9 (HFM+)	128.8±3.6	286.8±7.1	605.1±15.0	1173.3±23.3a	1833.9±37.2a
Significance[2]	NS	NS	NS	*	*

[1] Values are means and standard errors. Values within a column not having the same letter are significantly different.

[2] NS $P>0.05$, * $P < 0.05$.

Table 4 Mortality rates of the birds with different treatments (%)

Treatment	d1-7	d8-14	d15-21	d22-28	d29-35
1 (NFM-)	0.00±0.00	0.00±0.00	0.00±0.00a	0.67±0.67	2.00±0.89
2 (LFM-)	0.67±0.67	0.00±0.00	0.67±0.67ab	0.00±0.00	0.67±0.67
3 (HFM-)	0.67±0.67	0.00±0.00	0.67±0.67ab	0.00±0.00	1.33±0.84
4 (NFM+Cp)	3.33±1.91	0.67±0.67	0.67±0.67ab	0.00±0.00	0.00±0.00
5 (LFM+Cp)	0.00±0.00	0.67±0.67	0.67±0.67ab	2.00±1.37	2.00±1.37
6 (HFM+Cp)	2.00±1.37	0.00±0.00	0.67±0.67ab	2.00±1.37	0.67±0.67
7 (NFM+)	1.33±0.84	0.67±0.67	2.67±1.33ab	0.00±0.00	0.67±0.67
8 (LFM+)	0.67±0.67	0.67±0.67	9.33±2.23bc	1.33±0.84	0.67±0.67
9 (HFM+)	0.67±0.67	0.00±0.00	12.00±4.95c	0.67±0.67	4.00±1.79
Significance[2]	NS	NS	**	NS	NS

[1] Values are means and standard errors. Values within a column not having the same letter are significantly different.

[2] NS $P>0.05$, ** $P < 0.01$.

pH and short chain fatty acids

Prior to Cp challenge on 14d, the value of pH and the concentrations of volatile fatty acids in the ileum of the birds were significantly affected by the fishmeal feeding and/or *Eimeria* infection (Tables 5 and 6). The total concentration of short chain fatty acids was significantly higher in 50% fishmeal feeding group than in the 25% or no fishmeal groups ($P < 0.001$). Individually, the concentration of formic acid was significantly elevated in the 25% fishmeal group compared to the non fishmeal feeding group ($P < 0.01$). Interestingly birds fed 50% fishmeal did not show any significant difference with either low or non fishmeal feeding birds; there was no significant impact of fishmeal feeding on acetic acid; and 30% fishmeal feeding significantly increased the concentration of lactic acid compared with both low and non fishmeal feeding treatments. Fishmeal did not have any impact on the pH value in the ileum prior on 14d.

Table 5 pH and Concentration (mM) of total and individual short chain fatty acids in ileal content of birds on 14d fed different levels of fishmeal[1]

Treatment	pH	SCFA total concentration	formic acid	acetic acid	lactic acid
HFM	7.69±0.07	45.35±5.98b	0.75±0.08ab	1.62±0.13	34.21±4.11b
LFM	7.81±0.08	22.39±3.61a	0.94±0.12b	2.03±0.23	16.63±3.23a
NFM	7.70±0.07	17.56±2.45a	0.48±0.05a	1.55±0.1	12.11±2.09a
Significance	NS	***	**	NS	***

HFM: high fishmeal (50%); LFM: low fishmeal (25%); NFM: no fishmeal.

[1] Values are means and standard errors. Values within a column not having the same letter are significantly different.
[2] NS $P>0.05$, ** $0.01 > P > 0.001$, *** $P < 0.001$.

The ileal pH was significantly lower in the birds with *Eimeria* infection compared to the birds without *Eimeria* infection ($P < 0.001$). The total concentration of ileal volatile fatty acids was significantly higher in birds with *Eimeria* infection than those without *Eimeria* infection ($P < 0.05$). Ileal formic acid decreased ($P < 0.01$) while lactic acid increased ($P < 0.05$) in the birds with *Eimeria* infection compared to the non inoculated group. Acetic acid concentration did not show significant difference in two groups of birds however.

Table 6 pH and concentration (mM) of total and percentages of individual short chain fatty acids in ileal content of birds on 14d with or without *Eimeria* infection[1]

Treatment	pH ***	SCFA total concentration*	formic acid**	acetic acid	lactic acid*
Eimeria positive	7.44±0.06	38.02±6.51	0.56±0.12	1.66±0.25	26.74±4.26
Eimeria negative	7.89±0.04	23.64±2.71	0.80±0.06	1.77±0.08	18.11±2.55

[1] Values are means and standard errors. Asterisks show significance difference between the means in the column: * $0.05 > P > 0.01$, ** $0.01 > P > 0.001$, *** $P < 0.001$.

Following the Cp challenge on 17d, ileal pH was not significantly affected by treatment, whereas the concentrations of the short chain fatty acids changed significantly (Table 7). The concentrations of total SCFA showed significant differences between birds with different treatments ($P < 0.05$). The concentrations of total SCFA significantly increased in the ileum of LFM+ and HFM+ birds compared to the LFM- birds. A significant difference was also observed between the LFM+ and HFM+Cp. Individually, the concentrations of formic acid were significantly different among the groups of birds ($P < 0.01$), with the concentration in HFM+Cp birds higher than all unchallenged groups (NFM-, LFM- and HFM), NFM+ and LFM+ birds; the concentrations of acetic acid was higher in HFM+Cp than only LFM- group ($P < 0.05$); the concentrations of lactic acid was higher in the LFM+ group than in the LFM- and HFM+Cp groups ($P < 0.05$).

Table 7 pH and concentrations (mM) of total and individual short chain fatty acids in ileal content of birds on 17d with different treatments[1]

Treatment	pH ileum	SCFA total concentration	formic acid	acetic acid	lactic acid
1 (NFM-)	6.31±0.20	56.44±14.35abc	0.06±0.05a	2.92±0.47ab	51.11±13.73ab
2 (LFM-)	6.76±0.15	34.23±9.34a	0.18±0.09a	1.85±0.50a	30.21±8.76a
3 (HFM-)	6.65±0.28	62.48±14.20abc	0.11±0.07a	2.95±0.83ab	57.17±13.34ab
4 (NFM+Cp)	6.51±0.17	47.61±7.02abc	0.59±0.29ab	3.61±0.67ab	40.92±7.12ab
5 (LFM+Cp)	6.35±0.17	52.28±17.38abc	0.43±0.15ab	4.10±0.76ab	55.23±17.43ab
6 (HFM+Cp)	6.41±0.20	38.94±8.40ab	1.40±0.58b	5.33±1.20b	29.23±8.79a
7 (NFM+)	5.98±0.31	68.23±16.36abc	0.04±0.04a	4.20±0.71ab	61.60±15.87ab
8 (LFM+)	6.18±0.20	98.95±4.79c	0.12±0.08a	4.22±0.27ab	92.06±4.92b
9 (HFM+)	6.35±0.32	92.25±13.73bc	0.50±0.18ab	4.96±0.85ab	83.74±13.90ab
Significance	NS	*	**	*	*

[1] Values are means and standard errors. Values within a column not having the same letter are significantly different.

[2] NS P>0.05, * 0.05 > P > 0.01, ** 0.01> P > 0.001.

Enumeration of bacteria

Prior to Cp challenge on 14d of experiment, the bacterial numbers in ileal contents were analysed to assess their responses to feeding of different levels of fishmeal, and *Eimeria* infection (Table 8). No significant difference was observed for all the bacteria analysed in response to *Eimeria* infection, while fishmeal feeding significantly lifted the level of Cp according to both culture and real-time PCR methods ($P < 0.01$ and 0.05 respectively). The trends of the bacterial change were similar for both methods. The culture method showed that 50% and 25% fishmeal feeding significantly increased Cp levels in the ileum compared to the no fishmeal treatment, whereas the real-time PCR method indicated that the birds fed 50% fishmeal had a higher Cp level than birds fed 25% fishmeal and no fishmeal diets.

Table 8 Bacterial enumeration (log_{10}CFU) in ileal content of birds fed different levels of fishmeal on 14d[1]

Treatment	Total anaerobic	Entero-bacteria	LAB	Lacto-bacteria	Cp-count	Cp-qPCR
HFM	7.45±0.09	4.58±0.55	7.47±0.47	4.92±0.86	3.38±0.59b	2.55±0.46b
LFM	7.53±0.11	4.22±0.59	8.01±0.12	4.14±0.90	2.29±0.57b	1.36±0.24a
NFM	7.39±0.09	3.11±0.55	8.05±0.12	5.09±0.88	0.75±0.41a	1.22±0.30a
Significance	NS	NS	NS	NS	**	*

HFM: high fishmeal (50%); LFM: low fishmeal (25%); NFM: no fishmeal.

[1] Values are means and standard errors. Values within a column not having the same letter are significantly different.

[2] NS P>0.05, ** 0.01 > P > 0.001, *** P < 0.001

Following Cp challenge on 17d of the experiment, the treatments did not show impact on the growth of total anerobic bacteria, entero-bacteria, lactic acid bacteria, and lacto-bacillus in the ileum of the birds, while significant differences of Cp level were observed amongst the treatments according to both the culture technique and the real- time PCR method (Table 9). Based on culture technique, significantly higher Cp levels were found in the LFM+Cp birds compared to LFM- birds ($P < 0.01$). For the real-time PCR method, significant differences were observed among birds in several groups ($P < 0.001$). Compared to the NFM- and LFM-birds, the Cp levels of LFM+Cp, HFM+Cp, LFM+ and HFM+ birds significantly elevated. In both HFM+Cp and HFM+ groups, the Cp levels were significantly higher than all the unchallenged groups (NFM-, LFM- and HFM-). Among the challenged groups without *Eimeria* infection (NFM+Cp, LFM+Cp

and HFM+Cp), 50% fishmeal treatment significantly increased Cp level in comparison with no fishmeal treatment.

Table 9 Bacterial enumeration (log_{10}CFU) in ileal content of birds with different treatments on 17d[1]

Treatment	Total Anerobic	*Entero-bacteria*	Lactic acid bacteria	*Lacto-bacillus*	*Cp*-count	*Cp*-qPCR
1 (NFM-)	7.58±0.16	1.51±0.62	8.54±0.96	8.33±0.16	1.74±0.79ab	3.01±0.16a
2 (LFM-)	7.33±0.22	2.11±0.86	8.16±0.95	-	1.21±0.78a	3.14±0.31a
3 (HFM-)	7.68±0.13	2.32±0.95	8.38±1.04	8.10±0.26	1.72±0.77ab	3.89±0.16ab
4 (NFM+Cp)	7.29±0.2	1.42±0.58	8.22±0.9	-	3.09±1.10ab	4.54±0.66abc
5 (LFM+Cp)	7.62±0.08	0.72±0.29	8.46±0.72	8.14±0.21	5.82±0.52b	6.25±0.57cd
6 (HFM+Cp)	7.5±0.14	2.44±1	8.19±1.11	-	4.70±1.04ab	6.99±0.66d
7 (NFM+)	7.67±0.12	2.16±0.88	8.59±0.98	8.36±0.14	4.24±1.02ab	4.78±0.31abcd
8 (LFM+)	7.72±0.03	1.58±0.65	8.56±1.01	-	4.81±1.03ab	5.63±0.49bcd
9 (HFM+)	7.85±0.05	3.14±1.28	8.57±1	8.45±0.06	5.20±1.11ab	6.30±0.66cd
Significance	NS	NS	NS	NS	**	***

[1] Values are means and standard errors. Values within a column not having the same letter are significantly different.
[2] NS P>0.05, ** 0.01 > P > 0.001, *** P < 0.001

Comparison of Cp analysis using real-time PCR and culture methods

Standard curve for quantitative PCR analysis was constructed by plotting threshold cycles against the common logarithm of the Cp concentration (Fig. 1). The results showed that the real-time PCR reaction and detection systems were considerably reliable with $R^2 = 1.00$ and amplification efficiency = 0.96.

As shown in Fig, 2, while the change of log_{10}Cp among treatments briefly showed similar patterns between culture and quantitative PCR methods, the absolute values were different. However, the regression result showed that the real-time PCR method significantly correlated with the culture technique ($R^2 = 0.845$) (Fig. 3) indicating the validity of real-time PCR method to quantify Cp.

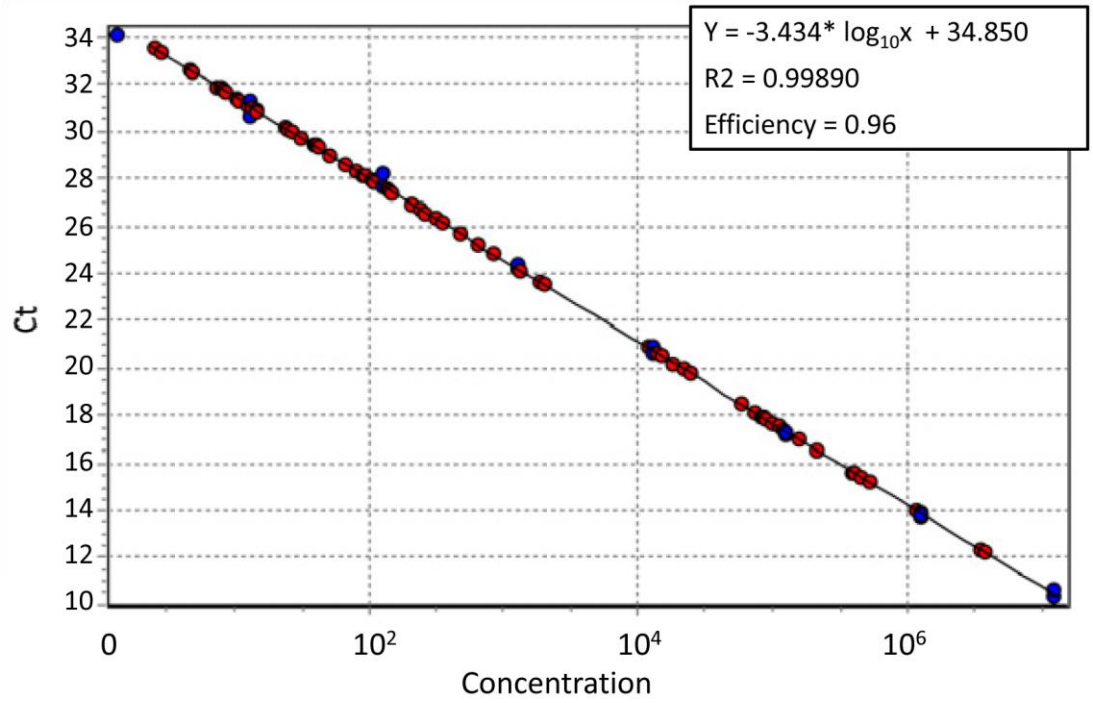

Fig. 1 Quantitative real-time PCR standard curve plotted by the threshold cycle (Ct) and the log_{10}(Cp concentration). ● samples with known concentrations of Cp; ● samples from experimental treatments on 17d.

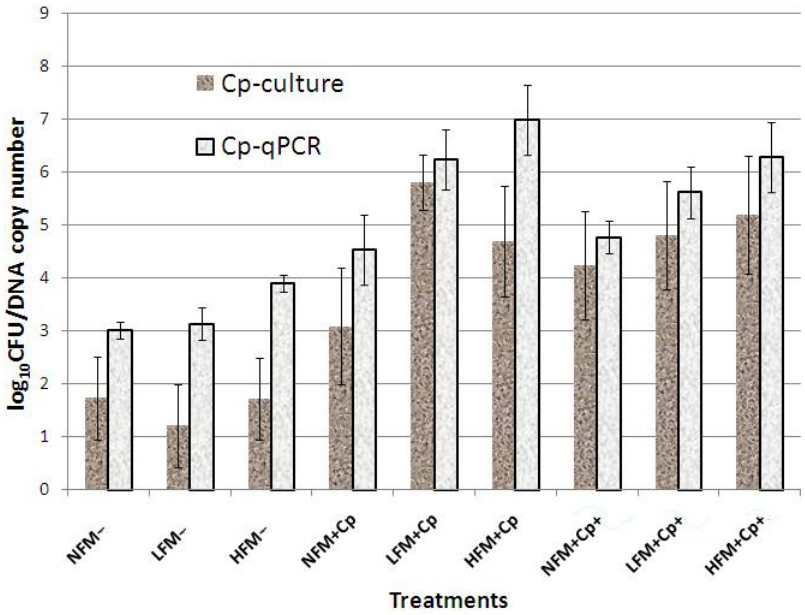

Fig. 2 Cp enumerations in response to experimental treatments measured by Cp culture and real-time PCR. Data are expressed as mean ± SE, n= 6.

To assess the accuracy of the real-time PCR method compared to the culture technique, relative standard deviations (RSD) of both methods were calculated, and the comparison is shown in Table 10. For all the treatments, the RSD of real-time PCR analysis were smaller relative to the culture method, indicating that the real-time PCR had higher accuracy than the culture technique. In the HFM- group, the RSD of the culture technique was as high as 10.8 times that of the real-time PCR method. The closest RSDs between these two methods were observed in LFM+Cp group, and interestingly the RSD of the culture technique in this group was the smallest among the RSDs of all the treatments measured by culture method. Birds without Cp infection had high RSDs for the culture technique largely because the Cp levels in the intestinal digesta may be lower than the detectable level. While the Cp counts increased after the challenge in other groups, the RSDs were still substantially high for the culture technique in contrast to the real-time PCR method.

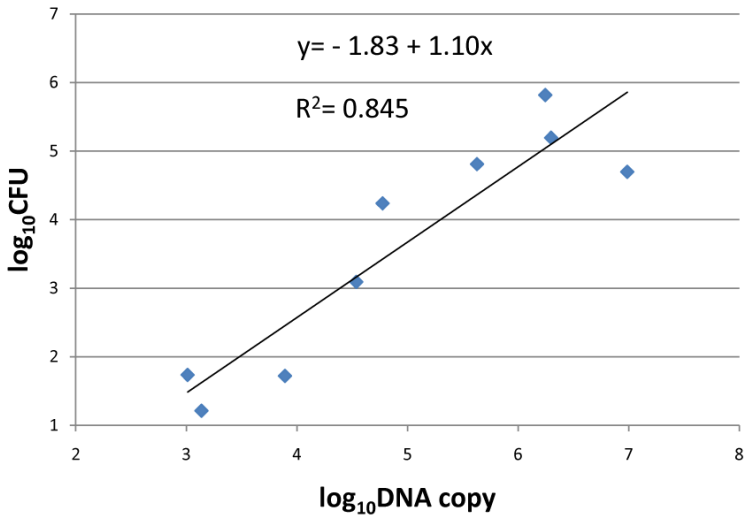

Fig. 3 Simple linear regression of $\log_{10}$DNA versus $\log_{10}$CFU of *C. perfringens* in the birds subjected to different treatments. Data are means of each treatment, n = 6.

Table 10 Comparison of relative standard deviations between culture and quantative PCR methods for bacterial enumeration in ileal content of birds on 17d with different treatments[1]

Treatment	Cp-count (%)	Cp-qPCR (%)	ratio counts to qPCR
1 (NFM-)	111.4	13.0	8.5
2 (LFM-)	158.1	24.2	6.5
3 (HFM-)	109.9	10.1	10.8
4 (NFM+Cp)	87.4	35.8	2.4
5 (LFM+Cp)	21.7	22.2	1.0
6 (HFM+Cp)	54.4	23.1	2.4
7 (NFM+)	58.7	16.1	3.7
8 (LFM+)	52.6	21.5	2.4
9 (HFM+)	52.5	25.8	2.0

Discussion

The main objective of this project was to determine whether *Eimeria* infection and fishmeal feeding are necessary for full NE challenge experiments, and whether quantitative real-time PCR could improve the accuracy and efficiency to enumerate bacteria in the intestinal tract of broiler chicken.

All together, nine experimental treatments were designed to investigate the effects of *Eimeria* infection and fishmeal feeding on the bird mortality and other factors such as bird weights, intestinal pH, concentrations of short chain fatty acids, and growth of bacteria prior to and following Cp challenge on 14d and 17d. The mortality results clearly indicated that infection of *Eimeria* and feeding of fishmeal diet were necessary to introduce NE. Intestinal damage caused by coccidial pathogens such as *Eimeria* species is known to be the most important predisposing factor to elicit the clinical signs and lesions of NE (van Immerseel *et al.*, 2004). As lack of a reproducible NE disease model has been a major obstacle in understanding the mechanisms of the disease, a reliable and reproducible model is essential and the conditions applied in the model must be very effective. According to the results obtained in this study, feeding of a diet containing 50% fishmeal and administration of *Eimeria* is the best combination to produce effective NE lesions in the gut of the birds challenged by Cp. Consequently, 50% fishmeal can be a recommended level to be used in NE challenge experiments. Although 25% fishmeal feeding in combination with *Eimeria* infection produced statistically higher mortality than only NFM- birds, the level of mortality (9.33%) is still acceptable for the Cp challenge experiment. Therefore, reducing the fishmeal level from 50% to 25% fishmeal in the diet for the NE challenge can be considered.

In addition to mortality, bird body weight, the concentrations of short chain fatty acids, and the levels of *C. perfringens* were affected by fishmeal feeding and *Eimeria* infection following Cp challenge. This may also be an indication of the necessity of feeding fishmeal diet and administration of *Eimeria* to promote NE in the chicken challenge model. Interestingly, while *Eimeria* proved to be essential in the NE challenge model, the Cp levels did not show any significant difference between the treatments with and without *Eimeria* infection following Cp challenge of the birds fed the same levels of fishmeal or no fishmeal feeding. This suggests that a high Cp level in the gut of birds does not necessarily translate into high mortality. Coccidiosis caused by *Eimeria* infection contributes to the severity of intestinal lesions which lead to the death of the birds, but do not alter the colonisation of Cp bacteria in the intestinal tract of broiler chickens. Conversely, a high Cp level in the gut that may be enhanced by fishmeal feeding could lead to subclinical infection of the birds. Additionally, the fact that Cp values of the birds with *Eimeria* infection was similar to that of the corresponding groups of the birds without *Eimeria* infection may be caused by random sampling of birds, as the birds with the highest Cp value may have died and did not have the opportunity to be analysed.

It has been reported that enumeration of intestinal bacteria using culture tedchniques has its limit and the culture-based process is extremely laborious (Dahiya *et al.*, 2007; Wise and Siragusa, 2007). Alternative methods including DGGE, TGGE, conventional PCR, and T-RFLP have been applied to analyse bacteria in the environment and animal gastrointestinal tract. However, these approaches are not fully quantitative. Therefore,

quantitative real-time PCR has been considered as a well-suited method to replace bacterial culture counting (Wise and Siragusa, 2007). Nevertheless, evaluation of these two methods has not been reported. In this study, we compared real-time PCR and bacterial culture methods to measure Cp in the ileum of the birds which were subject to nine treatments. It convincingly demonstrated that the real-time PCR method is more accurate than the conventional culture method, as it produced substantially smaller variation than the culture method. The accuracy of the culture method is usually restricted due to (a) the difference of the bacterial counting results by culture between undetectable and detectable replicates of a treatment can not be accurately estimated; (b) conventional bacterial culturing is a laborious procedure which can introduce systematic error; and (c) as the sample preparation of a culture-based method involves multiple technicians, other operator errors may also be introduced. In contrast, the real-time PCR method has high sensitivity that detects as low as a single copy of DNA and thus a single bacterial cell, and there is less chance of error. Hence, the possible variations occurred in a culture method are largely eliminated. Furthermore, the costs of real-time PCR are considerably lower than traditional culture methods. As estimated for an experiment involving 100 samples, and 5 bacterial species/groups to measure, the culture method costs about A$15 per bacterium/sample, whereas the real-time PCR costs about A$4 per bacterium/sample. Therefore, the real-time PCR method is not only efficient and accurate, but also cost-effective.

In conclusion, an NE challenge model of broiler chickens has been optimized by feeding the birds with 15-30% fishmeal and inoculating *Eimeria*, and real-time PCR detection of bacteria proved to be an efficient, accurate and cost-effective approach as an alternative for conventional culture method as demonstrated by the assay of Cp bacteria in the chicken ileum. Further studies should extend the current real-time PCR method to other bacterial groups.

Implications

Necrotic enteritis (NE) leads to over $2 billion annual loss of the world's broiler industry. An optimised NE challenge model will no doubt benefit the research community and industry. A reliable, efficient and cost effective bacterial quantification approach will also assist the researchers or industry to accurately and effectively conduct the analysis of the bacteria. It also has the potential for disease diagnosis in the broader community.

Recommendations

The NE challenge model optimised in the present study appears to be reliable and feasible for research community and industry. Other factors besides the focus of this study proved suitable for the NE challenge. However, the factors such as ambient temperature, diet formulation and timing of challenge may be further evaluated. Real-time PCR quantification of bacteria should be a direction to replace traditional culture based assay. The assay using real-time PCR to quantify other bacteria in addition to Cp in the NE challenge model should be assessed in future studies.

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