

# Natural plant extracts and prebiotic compounds as alternatives to antibiotics in broiler chicken diets in a necrotic enteritis challenge model

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**Abstract.** An experiment was conducted to determine the effects of two different water-soluble carbohydrate extracts (renga renga lily extract and *Acacia* extract), and two commercially available prebiotic compounds, Fibregum and Raftifeed-IPE, on the performance of broiler chickens subjected to a necrotic enteritis (NE) challenge model. These treatments were compared with negative control and a positive (Zn-bacitracin) control treatments. An overall 8.8% NE-related mortality was recorded, with mean jejunal and ileal lesion scores in dead birds ranging from 3.03 to 3.90 in all challenged groups except the positive control groups. NE-specific deaths or clinical abnormalities were not observed with unchallenged control and positive control groups. At 7 days post-challenge, the concentration of specific IgY antibodies against the  $\alpha$ -toxin of *Clostridium perfringens* in the serum was lower ( $P < 0.05$ ) in birds fed the positive control and Fibregum-supplemented diets than in the negative control group. However, birds fed Fibregum had increased ( $P < 0.05$ ) IgM concentration compared with those fed *Acacia* extract and lily extract. The Fibregum-fed group also had higher ( $P < 0.05$ ) IgA concentrations in serum than did the positive-control and lily extract-supplemented groups at 14 days but this effect did not persist to 21 days. The results from the present study demonstrated that supplementation with water-soluble carbohydrates from two plant sources was not effective in controlling NE. However, the prebiotic compound Fibregum was found to be having some immunomodulatory effects. Addition of Zn-bacitracin and monensin was highly effective in counteracting the negative effects of the disease challenge.

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## Introduction

Necrotic enteritis (NE) is an acute or chronic enterotoxaemia caused by *Clostridium perfringens* (van der Sluis 2003; Williams 2005). *C. perfringens* is a Gram-positive, obligate anaerobic, spore-forming bacterium readily found in soil, dust, used poultry litter and as a normal inhabitant of the gut microflora of healthy birds (Dahiya *et al.* 2006). Most often the only sign of an outbreak of clinical NE in broilers is a sudden increase in mortality which usually occurs between 2 and 5 weeks of age. The NE mortality rate within a flock is usually 2–10% but can be as high as 40–50% (Hofacre 2005). The proliferation of *C. perfringens* in the intestine and an increase in its toxin are considered the main cause of hemorrhagic necrosis of the intestinal mucosa (Dahiya *et al.* 2006). A subclinical form has been associated with reduced feed conversion efficiency and retarded growth rate in birds (Kaldhusdal and Hofshagen 1992). This disease has been reported in most areas of the world where broilers are produced under intensive management conditions, and it has a significant economic impact on the poultry industry (van der Sluis 2000).

A variety of in-feed antibiotics (IFAs), including virginiamycin, bacitracin, penicillin and tylosin, have been used in feed to effectively control and prevent NE (Watkins *et al.* 1997; Collier *et al.* 2003). However, many countries are moving towards a reduction in the use of IFAs in animal diets because large-scale use of antibiotics can cause resistant bacterial strains to develop (Barton 1998). As a consequence, the prophylactic use of most IFAs in poultry feed in Europe has been banned and there are increasing indications that other regions of the world will follow this trend (DANMAP 2008). However, this has resulted in problems such as increased mortality in poultry flocks, occurrence of ill-defined intestinal dysbacteriosis, a decline in bird welfare, an increase in the use of anticoccidial drugs and the incidence of food-borne human illness (Pattison 2002; Casewell *et al.* 2003). In the light of the situation where fewer, if any, antibiotics will be allowed in feeds in the future, it is important for the poultry industry to find alternative ways to control NE.

Several products have been investigated as potential alternatives to IFAs. For a product to be effective, it must

simulate one or more of the mechanisms by which IFAs function. It is believed that immune stimulation in meat-type chickens is important because these birds have lower antibody responses and non-specific proliferative responses than do layer-type strains (Koenen *et al.* 2004). McReynolds *et al.* (2004) reported that immunosuppression increases the severity of NE in broilers. Therefore, increasing the immunomodulating capacity of broilers to respond effectively to the diversity of antigens during early life is important. Guo *et al.* (2004) observed that mushroom and herb extracts may have significant impacts on the inductive immune responses against *Eimeria tenella* infection in broilers, by enhancing both cellular and humoral immunity. Prebiotic compounds such as oligosaccharides may also act as immunomodulators at the intestinal level. Kudoh *et al.* (1999) reported that Immunoglobulin A (IgA) secretion from caecal mucosa was promoted by orally administered highly fermentable, indigestible saccharides. Kleessen *et al.* (2003) reported that fructans-rich Jerusalem artichoke syrup administered via drinking water resulted not only in a significant reduction in the numbers of *C. perfringens* in caecal chyme, but also decreased the levels of microbial endotoxins in the blood of broilers. However, there is very little information available in relation to the influence of most of these prebiotic and bioactive substances on immune responses in chickens challenged with *C. perfringens* or *Eimeria* spp. In view of the growing interest in investigating potential alternatives for IFAs in poultry feed, testing novel forms of prebiotic and bioactive compounds is of utmost importance.

Previous feeding experiments conducted using caged broilers indicated that inclusion of prebiotic plant extracts beneficially modulated the composition of the microflora in the ileum and caeca, by increasing the number of lactobacilli and reducing harmful bacteria, such as potentially pathogenic coliforms and *C. perfringens* (Vidanarachchi *et al.* 2006, 2010b). Hence, it was hypothesised in the present study that dietary supplementation with *Acacia* extract, renga renga lily extract and commercially available prebiotic compounds with chemical composition similar to the plant extracts, namely, Fibregum and Raftifeed-IPE, would exert prebiotic effects and, thereby, control NE in birds challenged with *C. perfringens*. The objectives of the present study were to evaluate the effects of these prebiotic compounds on production performance, gut microflora composition, gut morphology and humoral immune responses of broiler chickens subjected to a NE disease challenge model involving oral inoculation with *C. perfringens*.

## Materials and methods

### *Birds and housing*

One thousand and fifty day-old male, commercial broilers (Cobb), vaccinated against Marek's disease, infectious bronchitis and Newcastle disease, were obtained from a local hatchery (Baiada hatchery, Kootingal, New South Wales (NSW), Australia). The research facility was thoroughly cleaned and disinfected before bird placement. One-day-old chicks were placed in 42 floor pens in a semi-commercial broiler shed located at the University of New England Kirby Research Station, Armidale, NSW, Australia. The pens were randomly assigned to seven treatments, each treatment being replicated six

times. Each pen (1.44 m<sup>2</sup>) was stocked with 25 chicks. The pens were enclosed with metal plates at the sides. A space of at least 4 m was maintained between the challenged and unchallenged bank of pens to avoid cross-contamination. The shed temperature was set at 33–34°C during the first week and gradually decreased by 3°C per week until 24–25°C was reached by the third week. Relative humidity was between 65% and 70%. Chicks were subjected to artificial fluorescent illumination of 23 h from 1 to 21 days of age, and of 18 h from 22 to 35 days of age. Each pen was equipped with a separate feeding trough and water was supplied through nipple drinkers. Water and feed were provided *ad libitum*.

### *Experimental diets*

The composition of the basal diets is shown in Table 1. The seven treatment groups were (1) an unchallenged group fed basal diet (UC; unchallenged control), (2) a challenged group fed basal diet (NC; negative control), (3) a challenged group fed basal diet supplemented with antibiotic (PC; positive control; Zn-bacitracin, 45 mg/kg) and coccidiostat (monensin, 100 mg/kg), (4) a challenged group fed basal diet supplemented with 10 g/kg water-soluble carbohydrates (WSCs) from golden wattle (*Acacia pycnantha*) exudate (*Acacia* extract, 76% (w/w) arabinogalactans), (5) a challenged group fed basal diet supplemented with 10 g/kg prebiotic arabinogalactan product Fibregum (Fibregum, Colloides Naturels International, Rouen Cedex, France), (6) a challenged group fed basal diet supplemented with 10 g/kg water-soluble carbohydrates from renga renga lily (*Arthropodium cirratum*) rhizomes (lily extract, 65% (w/w) inulins) and (7) a challenged group fed basal diet supplemented with 10 g/kg prebiotic inulin compound, Raftifeed-IPE (Raftifeed, Orafit Active Food Ingredients, Tienen, Belgium). The extraction and characteristics of WSCs from the two plant sources was described previously (Vidanarachchi *et al.* 2009). The plant extracts and prebiotic products were included as substitutes for wheat, and all supplements were added to the respective diets for the entire experimental period at the inclusion rates indicated above. Raftifeed-IPE contains mainly inulin (>700 g/kg fructo-oligosaccharides) extracted from chicory root and some glucose, fructose and sucrose (100 g/kg). The average chain length (degree of polymerisation) of the inulin in Raftifeed-IPE is ~2–60. Fibregum is a naturally occurring arabinogalactan extracted from the exudate of *Acacia senegal*. The Fibregum contains >900 g/kg arabinogalactans.

The basal diets were prepared commercially (Ridley Agriproducts, Tamworth, NSW, Australia) with the same batch of ingredients and bulk-shipped to University of New England. On arrival, the various supplements were properly mixed in according to the treatments, and then cold-pelleted (52–63°C).

### *Necrotic enteritis challenge*

From a day after hatching until 7 days of age, the birds were fed with starter diets. From Day 8 to Day 14 before inoculation with *C. perfringens*, the birds were fed a high-protein diet based on 50% (w/w) fish meal (with the full dose of supplements). After Day 14, the starter diets were returned until Day 21. The starter feed was replaced by the finisher feed on Day 22 and birds were

**Table 1. Ingredient and nutrient composition of experimental diets fed**

Vitamin mineral premix (Ridley Agriproducts, Tamworth, NSW) contained the following minerals in milligrams per kilogram of diet: Mn, 80; Zn, 60; Fe, 60; Cu, 8; I, 1.2; Co, 0.3; Se, 0.1; Mo, 1.0; and the following vitamin per kilogram of diet: Vitamin A, 12 000 IU from all *trans*-retinyl acetate; cholecalciferol D<sub>3</sub>, 3.500 IU; Vitamin E, 44.7 IU from DL- $\alpha$ -tocopherol; Vitamin B<sub>12</sub>, 12.75  $\mu$ g; riboflavin, 6.0 mg; niacin, 50 mg; pantothenic acid, 12 mg; folic acid, 2 mg; biotin, 0.1 mg; thiamine, 2 mg; Vitamin K, 2 mg and pyridoxine, 5 mg

Attribute	Starter (Days 1–7 and Days 15–21)	High-protein starter (Days 8–14)	Finisher (Days 22–35)
	<i>Ingredient (g/kg)</i>		
Wheat	475	342	500
Oats	100	100	100
Wheat offal	–	40	–
Rice pollard	26	–	26
Tallow	35	–	62
Soybean meal (48% crude protein)	190	–	150
Meat-bone meal	80	–	75
Peas	75	–	72
Fish meal	–	500	–
Limestone (38% Ca)	5.0	5.0	4.0
Sodium bicarbonate	3.4	3.4	2.0
Salt (NaCl)	1.0	1.0	1.5
Lysine–HCl	2.2	2.2	2.3
DL-methionine	3.3	3.3	2.5
L-threonine	1.0	1.0	0.3
Choline chloride	0.6	0.6	0.4
	<i>Premix – nutrient composition</i>		
Metabolisable energy (MJ/kg)	12.8	12.3	13.4
Crude protein (g/kg)	230	448	210
Crude fat (g/kg)	69	72	94
Lysine (g/kg)	14	32	11
Methionine (g/kg)	6	13	5
Available phosphorus (g/kg)	4	17	4
Calcium (g/kg)	10	25	10

kept until Day 35. On Day 9, all the birds, except those in the UCs, were given, *per os*, a suspension of 2500 oocysts of *Eimeria acervulina*, *E. maxima* and *E. tenella* in 1 mL of phosphate buffered saline (PBS). Unchallenged birds received sterile PBS. The attenuated *Eimeria* isolates originated from commercial broiler farms and were obtained from Bioproperties, Glenorie, NSW, Australia. The three species of *Eimeria* had been purified by serial passages through 3-week-old *Eimeria*-free chickens, and the sporulated oocysts were stored in 2% (w/v) potassium dichromate at 10°C before inoculation.

A primary poultry isolate of *C. perfringens* Type A was obtained from the CSIRO laboratory, Geelong, Victoria, Australia, and maintained in thioglycollate broth (USP alternative, Oxoid, Thermo Fisher Scientific Australia Pty Ltd, Adelaide, Australia) with 30% (v/v) glycerol at –20°C. The challenge inocula were prepared fresh by growing the bacterium overnight at 39°C in 1000 mL of thioglycollate broth with added starch (10 g/L) and casitone (5 g/L). The stock culture of *C. perfringens* had been previously subcultured in cooked meat media (Oxoid, CM81) and thioglycollate broth. On Days 14, 15 and 16, birds in challenged groups were individually inoculated *per os* with 1 mL of *C. perfringens* grown in thioglycollate broth at a concentration of  $3.5 \times 10^8$  colony forming units (CFU)/mL,

and birds in unchallenged cages were gavaged with 1 mL of sterile thioglycollate broth. Unchallenged birds were serviced first to lessen the likelihood of cross contamination and feed was withdrawn from all pens for 3 h on all days, before commencement of inoculation.

A gross pathologic diagnosis of NE in all dead birds and sampled birds was based on the presence of intestinal lesions typical of naturally occurring and experimentally induced NE, as described by Prescott *et al.* (1978) and Broussard *et al.* (1986). The small intestine from each bird was incised longitudinally and examined for evidence of gross necrotic lesions. Small intestinal lesions were scored according to the criteria of Prescott *et al.* (1978) with slight modifications as illustrated in Fig. 1. All birds were examined twice daily and all dead chickens were immediately collected for postmortem analysis.

#### *Growth performance, collection and processing of intestinal samples*

Weekly weight gain and feed intake per pen were measured and feed conversion ratio (FCR) was adjusted for mortality, calculated on a pen basis. Birds were observed twice daily for general health. All dead birds and culls (due to unhealthy condition) were weighed and necropsied.





**Fig. 1.** Gross appearance of the jejunal and ileum lesions, showing criteria for assigning necrotic enteritis scores. 0 = no gross lesions, normal intestinal appearance; 1 = thin walled or friable and few whitish plaques in the serosal surface (mild); 2 = thin-walled, focal necrosis or ulceration, small amounts of gas production (moderate); 3 = thin-walled, large patches of necrosis, gas-filled intestine, small flecks of blood (marked or severe); 4 = severe extensive necrosis, marked haemorrhage, excessive amounts of gas in the intestine (very severe).

On Days 14 and 21, 12 chickens per treatment were randomly selected for necropsy. The intestines were removed aseptically. To synchronise the feeding pattern of the birds, light was switched off for 2 h, followed by at least 1 h of light before the chickens were sacrificed. Subsequently, the chest cavity and the abdomen were opened and the small intestine was ligated and removed from the bird. The contents of the ileum and caeca were collected by gently finger-stripping the respective intestinal segments into plastic containers. About 0.2 g of ileal and caecal contents were suspended in 0.8 mL of distilled water, and the pH was measured with a glass pH electrode (EcoScan 5/6 pH meter, Eutech Instruments, Singapore) corrected for temperature. The remaining ileal and caecal digesta samples were frozen immediately after collection so as to measure short-chain fatty acid (SCFA) contents (only on Day 21). For histomorphological analysis, ~2.5 cm of the middle portion of the ileum was excised, flushed with PBS buffer (pH 7.6) and fixed in 10% (v/v) neutral buffered formalin. The bursa of Fabricius and spleen were removed from 12 birds of each treatment at Days 14 and 21 and weighed to the nearest gram. The data were expressed as a percentage of bodyweight.

#### Enumeration of bacteria and gut histomorphology

Samples in pre-reduced salt medium were homogenised for 2 min in CO<sub>2</sub>-flushed plastic bags using a MiniMix bag mixer (Interscience, St Nom, France) and serially diluted in 10-fold increments in pre-reduced salt medium according to the technique of Miller and Wolin (1974). An aliquot (100 µL) was plated on the following agar media. Total anaerobic bacteria were enumerated on Wilkins–Chalgren anaerobic agar (Oxoid, CM0619) after incubation at 39°C for 7 days in an anaerobic cabinet (Model SJ-3, Kaltec, Edwardstown, SA, Australia). Lactobacilli were enumerated on Rogosa agar (Oxoid, CM0627) after anaerobic incubation at 39°C for 48 h in anaerobic jars (Oxoid) with an anaerobic environment (<1% O<sub>2</sub> and 9–13% CO<sub>2</sub>), generated using anaerobic AnaeroGen sachets (AN0025A, Oxoid). 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 *C. perfringens* was determined on tryptose–sulfite–cycloserine and Shahidi–Ferguson perfringens agar base (TSC & SFP; Oxoid, CM0587) mixed with egg yolk emulsion (Oxoid, SR0047) and perfringens-selective (TSC) supplement (Oxoid, SR0088E) according to the pour-plate technique, where plates were overlaid with the same agar after spreading the inoculum. Bacterial numbers were expressed as log<sub>10</sub> CFU/g digesta. The morphology of the ileal tissues was determined as described by Vidanarachchi *et al.* (2010a).

#### Measurement of short-chain fatty acids and organic acids

To measure the concentration of SCFAs, lactic and succinic acids, ~2.0 g of thawed ileal and caecal sample were suspended in 1.0 mL of 0.02 M-2-ethylbutyric acid and thoroughly mixed by using a vortex mixer, followed by centrifugation at 25 700g at 4°C for 15 min. To a sample of 1 mL supernatant fraction, 0.5 mL of concentrated HCl and 2 mL of diethyl ether were added and thoroughly mixed by using a vortex mixer, followed by centrifugation at 2060g at

4°C for 15 min. Aliquots (360 µL) from the ether phase were recovered and mixed with 40 µL N-methyl-N-tert-butyltrimethylsilyltrifluoroacetamide for derivatisation of organic acids. The concentration of derivatised organic acids was quantified using a Varian CP 3400 CX gas chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA).

#### Enzyme-linked immunosorbent assay (ELISA)

##### Total serum antibody concentrations

Total antibody concentrations of IgY, IgM and IgA in serum were determined before *C. perfringens* challenge (14 days), and at 7 days after first challenge (21 days) using a sandwich ELISA (Bethyl Laboratories, Montgomery, TX, USA). Blood samples were collected from the jugular vein into 7-mL serum tubes and clotted at room temperature (RT; 25°C) for 2 h, and serum was separated from the cells by centrifugation at 2300g for 5 min and stored at –20°C.

##### Specific IgY antibodies against *C. perfringens* α-toxin

The specific IgY antibodies against the α-toxin of *C. perfringens* in blood serum were determined as described by Heier *et al.* (2001), with the following modifications: briefly, the microtitre plates were coated with 1 µg of phospholipase C Type I (7633; Sigma-Aldrich, St Louis, MO, USA) in 100 µL of carbonate buffer, pH 9.6 for 1 h at RT, washed three times with phosphate-buffered saline at pH 7.4 with 0.05% Tween 20 (PBST) and blocked with 200 µL of 1% bovine serum albumin in PBST for 1 h at RT. After washing as above, the plates were incubated with 100 µL of serum diluted to 1 : 250 in PBST, washed and incubated with 100 µL horseradish peroxidase-labelled goat anti-chicken IgY (Bethyl Laboratories, Montgomery, TX, USA) diluted to 1 : 200 in 1% bovine serum albumin in PBST for 1 h at RT. After washing, the colour reaction was developed with 100 µL of 3,3',5,5'-tetramethylbenzidine. The reactions were stopped by adding 100 µL of 2 M H<sub>3</sub>PO<sub>4</sub> and the absorbance was read at 450 nm using an ELISA microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA). The samples were analysed in duplicate and the results were expressed as average optical density values.

#### Statistical analyses

Bacterial counts were transformed to log<sub>10</sub> values, and molar proportions of SCFA and organic acids were subjected to arcsine transformation before statistical analysis; SCFA data are presented as natural numbers (Steel and Torrie 1981). Each variable (except mortality data and lesion scores) was analysed as a completely randomised design, with a pen of broilers composing an experimental unit. The experiment consisted of seven dietary treatments and data were analysed according to the following model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij},$$

where  $Y_{ij}$  = observed dependent variable,  $\mu$  = overall mean,  $\alpha_i$  = fixed effect of treatment,  $i = 1, 2, 3, 4, 5, 6, 7$ ,  $\beta_j$  = random effect of replicates,  $j = 1, 2, 3, 4, 5, 6$ ,  $\varepsilon_{ij}$  = residual error for Treatment  $i$  of Replicate  $j \sim N(0, \sigma_\varepsilon^2)$ .

All possible interactions were tested for significance (at  $P = 0.05$ ) and were eliminated from the models because they

**Table 2. Growth performance of broiler chickens fed diets supplemented with plant extracts and prebiotic compounds**  
Results are given as least-square means ( $n = 6$ ). s.e.m. = pooled standard error of the least-square means

Treatment	Bodyweight gain (BWG; g/bird)			Feed intake (FI; g/bird)			Feed conversion ratio (FCR)		
	Days 1–21	Days 21–35	Days 1–35	Days 1–21	Days 21–35	Days 1–35	Days 1–21	Days 21–35	Days 1–35
Unchallenged control (UC)	837	1068	1993	1177	2242	3551	1.41	2.10	1.78
Negative control (NC)	690	902	1741	1055	2065	3366	1.53	2.32	1.94
Positive control (PC)	805	1128	1998	1092	2113	3296	1.36	1.88	1.65
<i>Acacia</i> extract	725	969	1817	1099	2119	3611	1.52	2.20	1.99
Fibregum	713	941	1841	1069	2148	3534	1.50	2.29	1.92
Lily extract	692	882	1740	1087	2165	3533	1.57	2.48	2.03
Raftifeed	684	906	1712	1036	2066	3300	1.51	2.29	1.93
s.e.m.	14.35	36.71	44.47	23.59	48.71	112.43	0.02	0.09	0.05
<i>Orthogonal contrasts (probability level of contrasts)</i>									
UC vs NC	<0.0001	0.002	<0.0001	0.0003	0.01	n.s.	0.0002	n.s.	0.02
NC vs PC	<0.0001	<0.0001	<0.0001	n.s.	n.s.	n.s.	<0.0001	0.0005	<0.0001
NC vs <i>Acacia</i> extract	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NC vs Fibregum	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NC vs lily extract	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NC vs Raftifeed	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

were not significant. The errors were assumed to be independently and normally distributed, with a mean of zero and variance of  $\sigma_{\epsilon}^2$ . All data were analysed by using ANOVA option of PROC MIXED procedure (SAS Institute 2000), with treatment as the fixed effect and replicate as the random effect. Treatment least-squares means were compared using predetermined contrasts and considered significant at  $P = 0.05$ . Results are reported as least-squares means ( $n = 6$ ) and pooled standard error (s.e.). The mortality data were compared using a chi-square test.

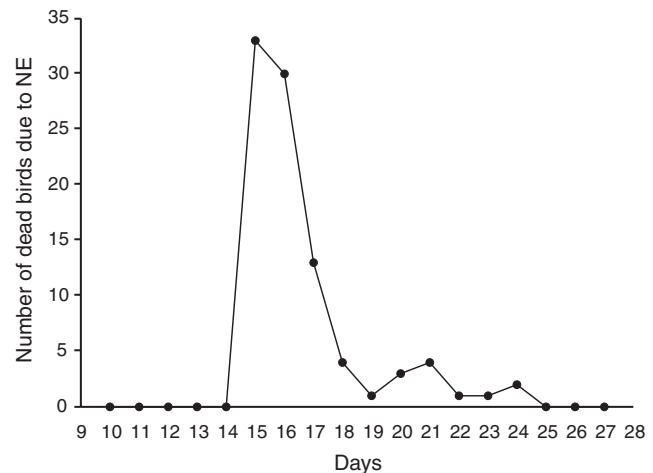
#### Animal ethics

All experimental procedures were approved by the University of New England Animal Care and Ethics Committee and throughout the experiments, health and husbandry practices complied with the *Code of Practice for the Care and Use of Animals for Scientific Purposes* (National Health and Medical Research Council 2004), for the Commonwealth of Australia and the *Australian Model Code of Practice for the Welfare of Animals: Domestic Poultry* (Primary Industries Standing Committee 2002).

## Results

#### Bird performance

The mean bodyweight gain (BWG), feed intake (FI) and FCR of chickens fed the experimental diets are shown in Table 2. There were no differences in growth performance between plant extract- or prebiotic-supplemented groups challenged with *C. perfringens* and the NC group during the entire experimental period. The BWG of challenged chickens on the control diet and plant extract (prebiotic) diets was markedly reduced compared with the UC group. This effect was most pronounced in the first 3 weeks. At the end of the 5-week period, BWG was decreased by 14% ( $P < 0.0001$ ) in the NC group compared with the UC group. Over the same period, FCR was impaired by 16 points due to *C. perfringens* challenge in the NC group. The FCR of the UC group was similar to that of broilers fed the PC diet during the 4th week of experiment. Over the whole



**Fig. 2.** Mortality pattern of broiler chickens challenged with *Clostridium perfringens*.

experimental period, there were no treatment effects on cumulative feed intake of birds. However, FI of challenged birds during the first 3 weeks was reduced ( $P < 0.0003$ ) compared with that of the UC group. The addition of Zn-bacitracin and monensin increased ( $P < 0.05$ ) average BWG and improved FCR throughout the experimental period. At 21 and 35 days, broilers on the PC diet were 17% and 15%, respectively, heavier than chickens from the NC group.

Mortality resulting from NE started at 1 day after birds were first challenged with *C. perfringens* (Fig. 2). Total NE-associated mortalities of 8.8% were observed for the whole experimental period. NE-associated mortalities occurred during the first 4 days and continued for a maximum of 7 days after the first challenge. NE-specific deaths or clinical abnormalities were not observed with UC and PC groups. The mean lesion scores in the jejunum in the challenged NC, *Acacia* extract, Fibregum, lily extract and Raftifeed groups were 3.18, 3.30, 3.34, 3.03 and

3.31, respectively. Corresponding values for the ileum were 3.18, 3.90, 3.36, 3.24 and 3.06, while mortality was 13.8%, 18.2%, 7.5%, 12.3% and 10.8%. Lesion scores were absent when the birds were sampled 7 days post-challenge.

### Gut histomorphology

Supplementation of the diets with plant extracts or prebiotic compounds had no effect on villus height or crypt depth of the ileum compared with the NC group on either sampling day (Table 3). At 14 and 21 days, the birds fed the PC diet had longer villi, shallower crypts and greater villus : crypt ratio in the ileum than did those in the NC group. However, there was no difference ( $P > 0.05$ ) in villus height of all prebiotic- and plant

extract-supplemented groups compared with the PC group before *C. perfringens* challenge. In the current study, the villus height and crypt depth of UC birds and NC birds were different ( $P < 0.05$ ) on Day 21 (after challenge). The histomorphological analysis of ileal tissues on Day 21 also revealed that detachment and disruption of the normal apical microvilli of enterocytes, which is characteristic of NE, was observed in two regions of the gut.

### Ileal and caecal microflora

The results of the bacterial counts in ileal and caecal contents are shown in Tables 4 and 5. In general, coliforms, lactose-negative enterobacteria and *C. perfringens* counts were higher in both

**Table 3. Effects of dietary plant extracts and prebiotic compounds on the morphometric parameters of ileum at Day 14 and Day 21**

Results are given as least-square means ( $n = 6$ ). s.e.m. = pooled standard error of the least-square means

Treatment	Before challenge (Day 14)			After challenge (Day 21)		
	Villus height ( $\mu\text{m}$ )	Crypt depth ( $\mu\text{m}$ )	Villus : crypt ratio	Villus height ( $\mu\text{m}$ )	Crypt depth ( $\mu\text{m}$ )	Villus : crypt ratio
Unchallenged control (UC)	611	82	7.54	705	75	9.36
Negative control (NC)	605	81	7.68	624	85	7.39
Positive control (PC)	702	62	11.54	720	73	9.92
<i>Acacia</i> extract	641	79	8.25	631	88	7.19
Fibregum	657	78	8.43	658	93	7.13
Lily extract	636	87	7.43	631	88	7.24
Raftifeed	664	75	8.93	663	91	7.34
s.e.m.	22.50	4.26	0.56	25.14	2.97	0.42
<i>Orthogonal contrasts (probability level of contrasts)</i>						
UC vs NC	n.s.	n.s.	n.s.	0.02	0.01	0.0001
NC vs PC	0.005	0.004	<0.0001	0.01	0.006	0.0002
NC vs <i>Acacia</i> extract	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NC vs Fibregum	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NC vs lily extract	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NC vs Raftifeed	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

**Table 4. Effect of dietary plant extracts and prebiotic compounds on ileal digesta bacterial counts ( $\log_{10}$  CFU/g digesta) of broiler chickens on Days 14 and 21**

Results are given as least-square means ( $n = 6$ ). s.e.m. = pooled standard error of the least-square means. Values for total anaerobic bacteria and lactose-negative enterobacteria were not significantly affected by treatment and were excluded from the table

Treatment	Lactobacilli		Coliform bacteria		<i>Clostridium perfringens</i>	
	Day 14	Day 21	Day 14	Day 21	Day 14	Day 21
Unchallenged control (UC)	7.19	8.23	8.76	7.99	9.00	6.11
Negative control (NC)	7.64	8.36	8.72	7.82	9.03	6.70
Positive control (PC)	6.66	7.44	8.75	7.59	3.53	3.33
<i>Acacia</i> extract	8.24	8.83	8.21	7.74	9.29	6.44
Fibregum	7.29	8.34	8.18	7.13	8.35	6.58
Lily extract	8.00	8.94	8.72	7.50	9.03	6.80
Raftifeed	8.08	8.90	8.90	7.56	9.04	6.74
s.e.m.	0.24	0.16	0.17	0.31	0.26	0.32
<i>Orthogonal contrasts (probability level of contrasts)</i>						
UC vs NC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NC vs PC	0.006	0.002	n.s.	n.s.	<0.0001	<0.0001
NC vs <i>Acacia</i> extract	n.s.	0.02	0.04	n.s.	n.s.	n.s.
NC vs Fibregum	n.s.	n.s.	0.02	n.s.	n.s.	n.s.
NC vs lily extract	n.s.	0.01	n.s.	n.s.	n.s.	n.s.
NC vs Raftifeed	n.s.	0.02	n.s.	n.s.	n.s.	n.s.



**Table 5. Effect of dietary plant extracts and prebiotic compounds on caecal digesta bacterial counts ( $\log_{10}$  CFU/g digesta) of broiler chickens on Days 14 and 21**

Results are given as least-square means ( $n = 6$ ). s.e.m. = pooled standard error of the least-square means. Values for lactose-negative enterobacteria were not significantly affected by treatment and were excluded from the table

Treatment	Total anaerobic bacteria		Lactobacilli		Coliform bacteria		<i>Clostridium perfringens</i>	
	Day 14	Day 21	Day 14	Day 21	Day 14	Day 21	Day 14	Day 21
Unchallenged control (UC)	10.78	10.25	8.28	9.29	9.51	9.02	7.89	6.44
Negative control (NC)	10.51	10.12	8.26	9.23	9.55	9.03	8.88	6.50
Positive control (PC)	10.07	9.99	8.36	8.67	9.28	8.57	3.50	3.87
<i>Acacia</i> extract	10.29	9.87	8.98	9.80	8.90	8.78	8.13	6.67
Fibregum	10.27	10.09	8.25	9.54	8.85	8.59	6.97	6.00
Lily extract	10.51	10.17	8.65	9.69	9.15	8.97	8.09	6.79
Raftifeed	10.31	9.96	8.71	9.42	9.27	8.95	8.13	6.36
s.e.m.	0.09	0.11	0.32	0.19	0.17	0.19	0.37	0.40
<i>Orthogonal contrasts (probability level of contrasts)</i>								
UC vs NC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NC vs PC	0.002	n.s.	n.s.	0.04	n.s.	n.s.	<0.0001	<0.0001
NC vs <i>Acacia</i> extract	n.s.	n.s.	n.s.	0.04	0.005	n.s.	n.s.	n.s.
NC vs Fibregum	n.s.	n.s.	n.s.	n.s.	0.003	n.s.	0.001	n.s.
NC vs lily extract	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NC vs Raftifeed	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

ileal and caecal contents at 14 days (before challenge) than at 21 days. Total anaerobic counts in caecal digesta were decreased ( $P < 0.05$ ) by  $\sim 0.5$  log units in birds fed PC versus NC, on Day 14. Compared with the NC group, no difference ( $P > 0.05$ ) was detected among various dietary treatment groups with respect to total anaerobic bacterial numbers in ileal and caecal contents on Day 21 (Table 5).

The *Lactobacillus* counts in ileal and caecal digesta on Day 14 tended to be higher in *Acacia* extract-, lily extract- and Raftifeed-supplemented treatment groups than in the NC group. Birds fed with the same supplements had higher ( $P < 0.05$ ) *Lactobacillus* counts in ileal digesta than those of the NC group on Day 21. Furthermore, birds supplemented with *Acacia* extract had higher ( $P < 0.05$ ) counts of lactobacilli in caecal digesta on Day 21 than did the birds in the NC group. The counts of lactobacilli decreased by 9.5- and 8.3-fold in the ileal digesta of PC versus NC groups at 14 days and 21 days, respectively.

Compared with the NC group, ileal and caecal lactose-negative enterobacteria numbers were not different between dietary treatment groups. Both *Acacia*- and Fibregum-fed groups had lower ( $P < 0.05$ ) coliform numbers in ileal and caecal digesta on Day 14. Neither the ileal nor caecal coliform counts at 21 days were affected by dietary supplementation of plant extracts or prebiotic compounds.

*Clostridium perfringens* counts in ileal and caecal digesta in all treatment groups, except in the PC group, before *C. perfringens*-challenge were higher than those after *C. perfringens* challenge. The mean *C. perfringens* count in the ileum decreased from 8.96 to 6.56  $\log_{10}$  cfu/g of digesta in all treatment groups 7 days after the first *C. perfringens* challenge. Corresponding values for caecal contents decreased from 8.02 to 6.46  $\log_{10}$  cfu/g of digesta. Fibregum decreased ( $P < 0.05$ ) the *C. perfringens* numbers in caecal contents before *C. perfringens* challenge but this effect was not significant after *C. perfringens* challenge, although the values tended to be lower in that group than in the NC

group. Raftifeed and the two plant extracts did not significantly ( $P > 0.05$ ) affect *C. perfringens* populations before and after *C. perfringens* challenge. The *C. perfringens* counts in luminal contents from the ileum and caeca of birds fed the PC diet were significantly ( $P < 0.001$ ) lower than those from the NC groups at both sampling days.

#### *Organic acids and pH*

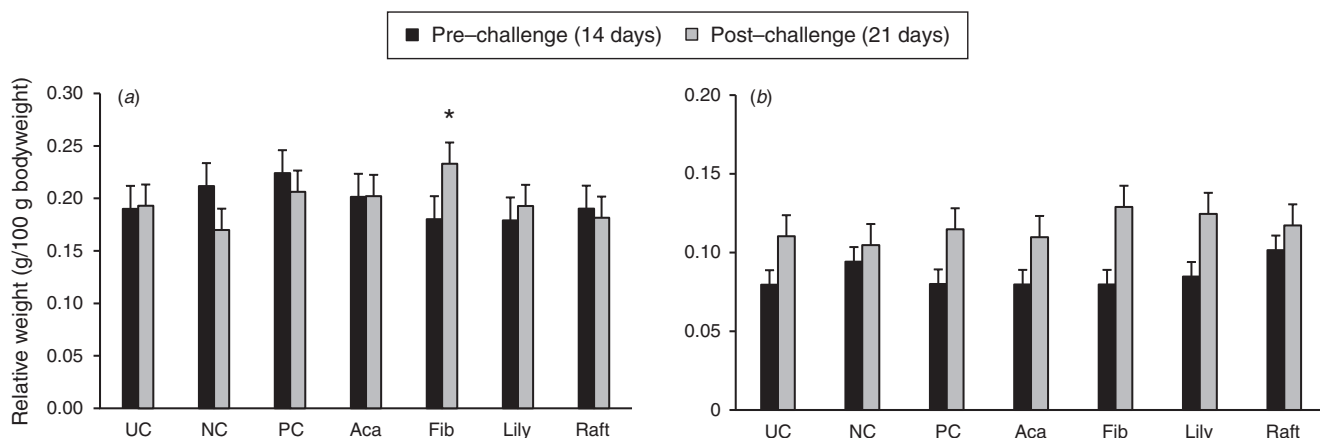
There were no major effects of treatments on ileal and caecal pH, concentration of total organic acids and molar proportions of acetic and lactic acids assessed at 21 days of age (data not shown). However, the pH ( $P < 0.002$ ) and molar proportion of acetic acid ( $P < 0.008$ ) in the ileal digesta of birds on the PC diet were higher than those on the NC diet. Conversely, birds on the NC diet had a higher ( $P < 0.01$ ) content of lactic acid in the ileal digesta than those on the PC diet. Similarly, the pH of the caecal digesta of birds on the NC diet was higher than that of birds on the *Acacia* extract ( $P < 0.0001$ ) and Raftifeed ( $P < 0.007$ ).

#### *Lymphoid organ weights and humoral immune responses*

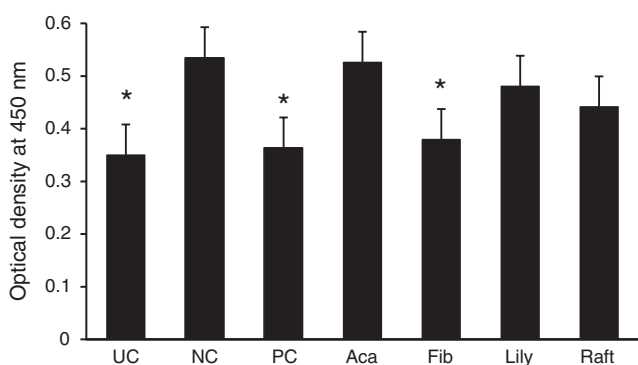
There were no differences in relative weights of spleen or bursa as a result of supplementation by either of the two plant extracts or of Raftifeed on either sampling day (before and after challenge; Fig. 3). However, the relative weight of bursa of birds supplemented with Fibregum was higher ( $P < 0.05$ ) than that of the NC group on Day 21.

Figure 4 shows the serum optical-density values of the specific serum IgY antibodies against  $\alpha$ -toxin (Phospholipase C) analysed by ELISA technique. At 7 days post-challenge with *C. perfringens*, the concentration of the specific IgY antibodies against the  $\alpha$ -toxin of *C. perfringens* in the serum, as represented in optical-density values (Fig. 4), was significantly ( $P < 0.05$ ) lower in birds fed with UC, PC and Fibregum diets than was the





**Fig. 3.** The effects of plant extracts and prebiotic compounds on relative weight of (a) bursa and (b) spleen of broiler chickens at Day 14 (before *Clostridium perfringens* challenge) and at Day 21 (after *C. perfringens* challenge) for unchallenged control (UC), negative control (NC), positive control (PC), *Acacia* extract (Aca), Fibregum (Fib), lily extract (Lily) and Raftifeed (Raft). Results are given as least-square means,  $n = 6$ ; error bars indicate pooled standard error of the least-square means; bars with asterisks are significantly different from the negative control.



**Fig. 4.** The effects of plant extracts and prebiotic compounds on specific IgY antibody titres against *Clostridium perfringens*  $\alpha$ -toxin at Day 21 (after *C. perfringens* challenge) for unchallenged control (UC), negative control (NC), positive control (PC), *Acacia* extract (Aca), Fibregum (Fib), lily extract (Lily) and Raftifeed (Raft). Results are given as least-square means of optical density values,  $n = 6$ ; error bars indicate pooled standard error of the least-square means; bars with asterisks are significantly different from the negative control.

concentration from the NC group. The total antibody responses to *C. perfringens* before and after challenge are shown in Fig. 5. The serum IgY response was not different among treatments before challenge. However, birds fed Fibregum had a higher IgM response than did birds in the *Acacia* extract-supplemented ( $P < 0.05$ ) and lily extract-supplemented ( $P < 0.05$ ) groups. The Fibregum-fed group also had higher ( $P < 0.05$ ) concentrations of IgA in serum than did the PC and lily extract-supplemented groups at 14 days, but this effect did not persist at 21 days. The concentration of IgY antibody titres in sera from the PC group was lower ( $P < 0.05$ ) than that in the NC group on Day 21.

## Discussion

### Bird performance

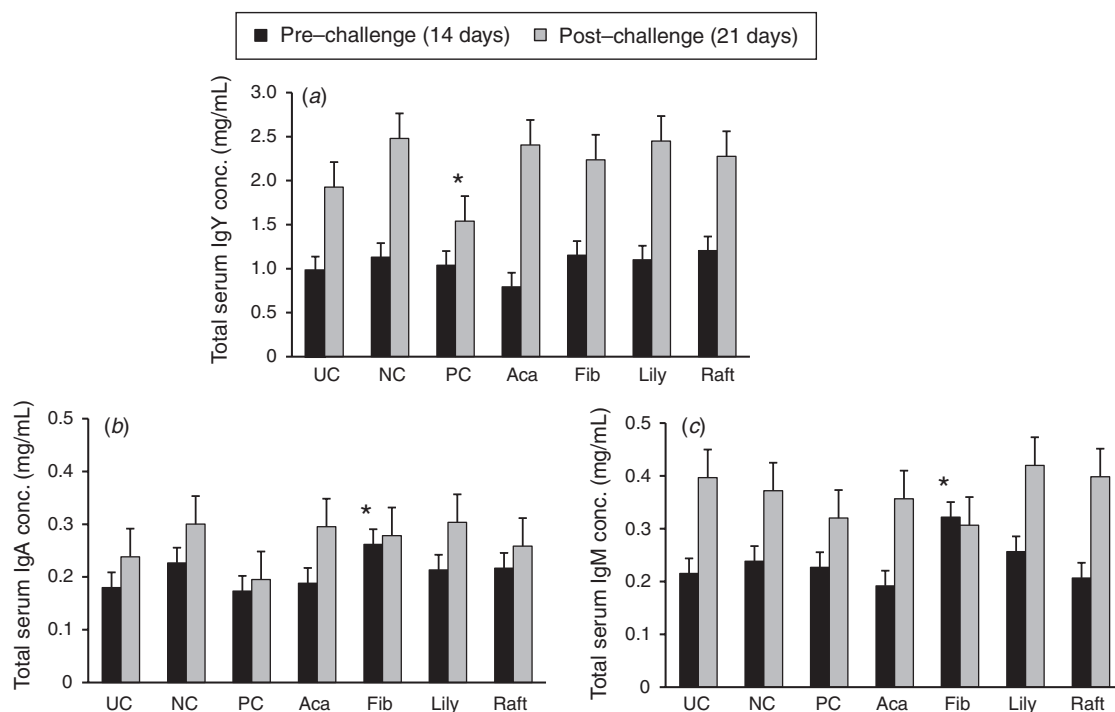
In the current study, except in the PC group, there was a significant decline in the growth performance of birds challenged with

*C. perfringens* in all treatment groups compared with unchallenged birds. The impaired FCR observed with all *C. perfringens*-challenged groups can be explained by decreased FI and BWG in the first 3 weeks of the period. Hofacre *et al.* (1998) and Dahiya *et al.* (2005) also reported decreased BWG and impaired feed conversion in broiler chickens challenged with *C. perfringens*. None of the test plant extracts or prebiotic products prevented the outbreak of NE or improved growth in a NE challenge model. These results are in agreement with the findings of Hofacre *et al.* (2003) who found no effect of fructo-oligosaccharide on growth performance or reduction in lesion scores in *C. perfringens*-challenged broilers. In contrast, Butel *et al.* (2001) reported that *C. perfringens*-induced necrotising enterocolitis in gnotobiotic quails could be controlled by dietary supplementation with oligofructose.

The Zn-bacitracin and monensin supplementation successfully prevented an outbreak of NE, while mortality due to NE occurred in spite of all the other supplements. Birds on Fibregum showed lower mortality from NE than those fed the *Acacia* extract, which had the highest mortality due to NE. High mortality of birds at 24 h post-challenge suggested that the experimental disease had a rapid onset in challenged animals and continued for a maximum of 7 days after the first challenge began. The lack of confluent necrosis and sloughing of the epithelial lining of the jejunum and ileum among the treatment groups was most likely influenced by the very low mortality among the challenged treatment groups 7 days post-challenge.

### Gut histomorphology

Our initial hypothesis was that plant extracts and prebiotic compounds in broiler diets challenged with *C. perfringens* would preserve the villus structure and villus length. However, the results showed that villus length was not affected by the plant extracts or prebiotic compounds. Dietary supplements such as prebiotic and bioactive compounds have been shown to have a trophic effect on the gut mucosal morphology in chickens (Iji *et al.* 2001; Yamauchi *et al.* 2006). It should be noted that these prior studies assessed compounds under



**Fig. 5.** The effects of plant extracts and prebiotic compounds on total serum immunoglobulin (a) IgY, (b) IgA, and (c) IgM titres in broiler chickens at Day 14 (before *Clostridium perfringens* challenge) and at Day 21 (after *C. perfringens* challenge) for unchallenged control (UC), negative control (NC), positive control (PC), *Acacia* extract (Aca), Fibregum (Fib), lily extract (Lily) and Raftifeed (Raft). Results are given as least square means,  $n = 6$ ; error bars indicate pooled standard error of the least-square means; bars with asterisks are significantly different from the negative control.

hygienic husbandry practices and not in a disease-challenged condition as was the case of the present study. However, fructo-oligosaccharides have been observed to alleviate salmonella-induced necrosis of the caecal mucosa and enhance the ileal micro-villus length of broilers (Choi *et al.* 1994).

In the current study, birds fed with the PC diet had longer villi and shallower crypts in the ileum, thus increasing the villus : crypt depth ratio, both before and after challenge. Supplementation of broiler diets with Zn-bacitracin and monensin effectively reduced the numbers of *C. perfringens* in the ileal and caecal contents during the experimental period. Hence, the damage that would have been caused to the intestinal mucosa by NE may have been prevented. Such morphological changes in Zn-bacitracin- and monensin-fed birds may also be responsible for the improved growth performance as seen in the current study in the disease-challenged groups. Antibiotic-treated farm animals are known to have longer villi and villus : crypt depth ratios in the small intestine than are their antibiotic-free counterparts (Nousiainen 1991; Ao 2004).

#### Ileal and caecal microflora

Although high *C. perfringens* counts (6.5–7.0 log<sub>10</sub> cfu/g) were observed in ileal and caecal digesta, focal necrotic lesions in the jejunum and ileum were not observed 7 days post-challenge. This demonstrated that a high population count of *C. perfringens* may not always be associated with subclinical or clinical NE due to the fact that it is a complex, multifactorial disease with

many unknown factors (Kaldhusdal *et al.* 1999; van Immerseel *et al.* 2004). Another explanation could be the TSC & SFP agar medium used in the present study may also have supported the growth of physiologically similar clostridia species resembling *C. perfringens*, as suggested by Adams and Mead (1980).

In the present study, dietary supplementation with two plant extracts (*Acacia* extract and lily extract) and the prebiotic product Raftifeed significantly increased or tended to increase the number of lactobacilli in the ileum at both sampling periods. These results are consistent with other work performed with plant extracts (Vidanarachchi *et al.* 2010b). Several mechanisms may account for the enhanced growth of lactobacilli in response to dietary supplementation with plant extracts. Certain dietary carbohydrates such as fructo-oligosaccharides and arabinogalactans are not digested in the small intestine, since the chicken lacks the enzymes to hydrolyse them (Xu *et al.* 2003; Saeed *et al.* 2011). Therefore, these carbohydrates are more likely to be degraded by the gastrointestinal microflora, which they selectively stimulate. Al-Tamimi *et al.* (2006) found that fermentation of arabino-oligosaccharides in a batch-culture fermentation system increased the lactobacilli and bifidobacteria counts, while decreasing *C. perfringens* numbers.

In the present study, feeding of *Acacia* extract or prebiotic compound Raftifeed caused reduction in coliform counts in the ileal and caecal digesta on Day 14, before *C. perfringens* challenge. The *Lactobacillus* counts in the same groups

were significantly higher or tended to be higher than in the NC group. Many studies have demonstrated a relationship between an increase in numbers of lactobacilli and decline in numbers of coliforms in chickens (Watkins *et al.* 1982; Jamroz *et al.* 2005).

As expected, the present study demonstrated that supplementation of broiler diets with Zn-bacitracin and monensin significantly reduced the *C. perfringens* counts in ileal and caecal digesta, with a consequent significant improvement in growth performance and pathophysiological indices of NE. The counts of *C. perfringens* in luminal contents from the ileum and caeca of birds fed the PC diet were as low as 3–4 log<sub>10</sub> cfu/g digesta on both sampling days, indicating that the IFAs used in the present study were highly effective in controlling *C. perfringens* proliferation in the gut of broilers. These findings are consistent with previous studies of the culture-based and molecular studies, which demonstrated that dietary supplementation with antibiotics such as Zn-bacitracin, bacitracin methylene disalicylate and tylosin causes a significant reduction in lesion scores and mortality caused by NE (Brennan *et al.* 2003; Collier *et al.* 2003).

#### Organic acids and pH

The supplementation of broiler diets with Zn-bacitracin and monensin in the present study significantly increased or tended to increase pH of ileal and caecal digesta. Correspondingly, the same treatment group had the lowest total organic acids and molar proportion of lactate in the ileal digesta. Since the PC group had significantly lower *Lactobacillus* counts, compared with the challenged control group (NC), it can be speculated that low microbial fermentation activities in the ileal digesta resulted in less volatile fatty acid production. Engberg *et al.* (2000) also observed higher pH and lower lactate concentrations in ileal digesta of broilers fed diets supplemented with salinomycin alone or salinomycin and Zn-bacitracin. The present results also confirmed those of Engberg *et al.* (2002) who found that lactate and acetate are the predominant organic acids produced due to bacterial fermentation in the ileum.

#### Lymphoid organ weights and humoral immune responses

The increase in the relative weight of the bursa by Fibregum treatment could be associated with an improvement in immune responses in broilers after *C. perfringens* challenge. The results of the present study are in agreement with the findings of Kleessen *et al.* (2003) who observed an increase in the relative weight of bursa of broilers in response to the consumption of Jerusalem artichoke fructans syrup (0.5%) administered via drinking water. Chen *et al.* (2003) noted a significantly larger bursa and greater immunomodulatory effect in broilers fed diets supplemented with two Chinese herbal polysaccharides, astragalum and achyranthum.

Birds from the UC, PC and Fibregum-supplemented treatment groups had lower serum concentrations of IgY antibody against *C. perfringens*  $\alpha$ -toxin and less mortality due to *C. perfringens*-associated NE. This indicated that antigenic stimulation was lower in these three groups. In agreement with this, *C. perfringens* counts in ileal and caecal digesta from the same three groups were either significantly lower or tended to be lower

than those of the other *C. perfringens*-challenged treatment groups. These immunological findings are in accordance with those of Løvland *et al.* (2003), who observed a higher level of IgY antibody against *C. perfringens*  $\alpha$ -toxin in the serum of broilers with a subclinical form of NE or *C. perfringens*-associated hepatitis. Detection of IgY antibody against *C. perfringens*  $\alpha$ -toxin in birds in the UC group may be due to a natural exposure of these birds to *C. perfringens* from the pen environment. The same group also had *C. perfringens* counts between 6.00 and 6.44 log CFU/g digesta in both ileal and caecal contents 7 days post-challenge. Løvland *et al.* (2003) also suggested that birds may become seropositive without exhibiting any *C. perfringens*-associated enteric gut lesions.

Results from the current study showed that Fibregum elevates serum IgM and IgA concentrations and enhances systemic immune capacity in chickens. The exact reason for this observation is not clear and further work on the determination of cell-mediated immune responses such as the proportions of different subpopulations of B and T lymphocytes is warranted. Elevated concentrations of serum IgA have been shown to correlate well with higher secretory IgA in the intestine (Brito *et al.* 1993) and may explain the mechanism of *C. perfringens* reduction from the intestinal lumen in the Fibregum-fed group. In conclusion, the results of the study suggested that neither the plant extracts nor the commercial prebiotic product were effective in controlling NE. The present study also showed no effect of dietary plant extracts on humoral immunomodulation in broiler chickens challenged with *C. perfringens*. Although some beneficial effects were observed with Fibregum, the product did not improve performance parameters, as BWG and FCR were similar to those for the NC. Under the conditions of the present study, Fibregum was effective in reducing NE-associated mortalities and in improving some immune response to the NE infection in broiler chickens. Dietary supplementation with Zn-bacitracin and monensin was highly effective in counteracting the negative effects of the disease challenge.

#### Acknowledgement

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