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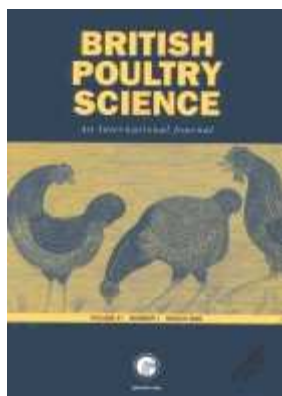
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Effect of trypsin inhibitor activity in soya bean on growth performance, protein digestibility and incidence of sub-clinical necrotic enteritis in broiler chicken flocks

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Running title: Necrotic enteritis and trypsin inhibitor

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2
3 **Abstract.** 1. The effect of three different levels of dietary trypsin inhibitor activity (achieved
4 by varying the amount of non-toasted full fat soya bean in replacement for toasted full fat
5 soya bean) on the incidence of spontaneously-occurring sub-clinical necrotic enteritis (NE) in
6 broiler chickens was compared. A fourth dietary treatment compared the effect of a diet that
7 used potato protein concentrate as the major protein source. The determined trypsin inhibitor
8 activity increased with the increasing content of non-toasted soya bean: 1.90, 6.21, 8.46 and
9 3.72 mg/g for the three soya bean diets (0, 100 and 200 g of non-toasted soya bean/kg) and
10 the potato protein diet respectively.
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22 2. Although increasing amounts of the non-toasted full-fat soya bean increased the feed
23 intakes of the birds, there was a marked reduction in protein digestibility, weight gain and
24 feed conversion efficiency.
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30 3. There was a linear increase in sub-clinical NE lesions in the duodenum, jejunum, mid
31 small intestine and ileum with increasing non-toasted soya bean. Caecal *Clostridium*
32 *perfringens* counts increased with the increasing dietary content of non-toasted soya bean.
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34 Serum α -toxin antibodies were higher in the birds fed the 200 g non-toasted soya bean/kg diet
35 compared with the other diets.
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41 4. The results demonstrated that variation in the amount of non-toasted dietary soya bean not
42 only affects growth performance of broilers but also affects the incidence of sub-clinical
43 necrotic enteritis in the flock. Ensuring the lowest possible trypsin-inhibitor activity in soya
44 bean samples is a valuable tool to improve the health and welfare of birds and in reducing the
45 financial losses from this disease.
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23 INTRODUCTION

24 Subclinical necrotic enteritis (NE) is an economically important bacterial disease in modern
25 broiler flocks (Kaldhusdal, 2000). The condition is not usually detected owing to the absence
26 of clear clinical signs and persists unnoticed and untreated. However, the disease may be
27 suspected when there are poor growth (Lovland and Kaldhusdal, 2001) and wet litter
28 conditions (Williams, 2005). Sub-clinical NE also increases the risk of contamination of
29 poultry products for human consumption (Craven *et al.*, 2001). The financial cost of the
30 disease to the world's poultry industry has been estimated as US\$2.6 billion per year (van der
31 Sluis, 2000). The causative organism, *C. perfringens*, is ubiquitous in the environment and the
32 intestines of most healthy animals and humans (Wages and Opengart, 2003). *C. perfringens* is
33 also a commensal bacterium of chicken intestines (Ewing and Cole, 1994) and there are
34 further predisposing factors that alter the intestinal balance to favour its proliferation and
35 toxin production. Intestinal *C. perfringens* and alpha-toxin levels (Hofshagen and Stenwig,
36 1992; Si *et al.*, 2007) are increased in the disease, so causing major pathological changes in
37 the small intestine (Gholamiandehkordi *et al.*, 2007) and liver (Lovland and Kaldhusdal,
38 2001).

39 NE has been reproduced experimentally by oral or intra-duodenal administration of
40 birds with pathogenic strains of *C. perfringens* isolated from clinically diseased birds
41 (Gholamiandehkordi *et al.*, 2007; Pedersen *et al.*, 2008). The majority of these experiments
42 have involved regular administration of the pathogen into a small number of birds kept in
43 cages (Drew *et al.*, 2004; Gholamiandehkordi *et al.*, 2007). These conditions are different
44 from practical broiler production methods. However, Lovland *et al.* (2003) found that sub-
45 clinical NE would develop spontaneously (without dosing the birds with pathogenic *C.*
46 *perfringens*) if appropriate predisposing factors (un-medicated diets, putative predisposing
47 feeding regimens, housing birds on litter) were provided. This method of reproducing sub-

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3 48 clinical NE provides the possibility of studying the effects of production variables under
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5 49 conditions that are directly related to commercial production methods.
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8 50 Apajalathi *et al.* (2001) identified the diet as the strongest determinant of the caecal
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10 51 bacterial community, so diet composition may influence the susceptibility of broilers to sub-
11
12 52 clinical NE. There is evidence that different dietary protein sources affect the proliferation of
13
14 53 *C. perfringens* within the caecum (Drew *et al.*, 2004) and in the ileum (Wilkie *et al.*, 2005)
15
16 54 when birds are orally dosed with these bacteria. The effect of different protein concentrates on
17
18 55 the incidence of spontaneously-occurring sub-clinical NE was studied in an earlier experiment
19
20 56 and there was a higher incidence of NE in birds fed potato protein-based diets compared with
21
22 57 soya bean-based diets (Palliyeguru *et al.*, 2010). High trypsin inhibitor activity (TIA) in this
23
24 58 diet was identified as a probable causative factor.
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29 59 Soya bean meal is the most common protein concentrate used in proprietary poultry
30
31 60 feeds. Soya bean must be heat-treated to improve protein digestibility due to the presence of a
32
33 61 heat-labile trypsin inhibitor. Heat denatures the trypsin inhibitor, but the process has a
34
35 62 variable efficiency that depends on the initial concentration of TIA, the moisture content of
36
37 63 the seeds, the temperature and time of heating (Liener, 1994). Precise control of heating
38
39 64 conditions is needed to prevent under or over-heating of the soya bean (Liener and Kakade,
40
41 65 1980). TIA has been identified as the best *in vitro* predictor of the nutritional value of
42
43 66 processed full-fat soya bean for chickens (Perilla *et al.*, 1997).
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48 67 A survey of commercially-available soya bean meals found there to be varying levels
49
50 68 of TIA (0.4-6.8 mg/g) (Probert, 2004) even though there is a European Union import
51
52 69 threshold of TIA for soya bean meal of 4 mg/g. Therefore, the quality of soya bean meal is a
53
54 70 factor that could affect the protein digestibility of the overall diet. Perilla *et al.* (1997) showed
55
56 71 that a TIA of 2.8 mg/kg of diet was associated with poor growth performance in broilers. Hill
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58 72 *et al.* (1957) demonstrated that the lower growth performance of broilers fed an unheated soya
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3 73 bean (100 g/kg of diet) could be eliminated by the addition of dietary antibiotics. This
4
5 74 indicates that small changes in TIA may affect the gut microflora of broiler chickens.
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8 75 The objective of this experiment was to compare the effect of three different levels of
9
10 76 TIA (achieved by varying the amount of unheated full fat soya bean in replacement for heat-
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12 77 treated full fat soya bean of the same original source) in nutritionally-complete diets, on the
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14 78 incidence of spontaneously-occurring, sub-clinical NE. A fourth dietary treatment compared
15
16 79 the effect of a diet that used potato protein concentrate as the major protein source. All 4 diets
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18 80 had nutrient compositions typical of those fed to commercial broilers.
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23 82 MATERIALS AND METHODS

24 83 Dietary treatments and experimental design

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28 84 Four nutritionally complete (Aviagen, 2007) maize-based, broiler-grower diets were
29
30 85 formulated. All 4 diets were formulated to have similar calculated contents of metabolisable
31
32 86 energy (13.1 MJ/kg), crude protein (210 g/kg), lysine (13 g/kg), methionine and cysteine (10
33
34 87 g/kg) and threonine (10 g/kg) (Table 1). Celite (20 g/kg) was included in each diet as an
35
36 88 indigestible marker for determining nutrient digestibility. Three increasing inclusion rates (0,
37
38 89 100 or 200 g/kg) of unheated full-fat soya bean (with high TIA) were included in replacement
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40 90 for a toasted full fat soya bean (from the same batch and with the same proximate
41
42 91 composition). A nutritionally-complete diet that included potato protein concentrate was also
43
44 92 formulated (Table 1). No antibiotic growth promoters or anti-coccidial drugs were used in the
45
46 93 diets. The formulated diets were mixed and steam-pelleted (3 mm diameter pellets). Chemical
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48 94 analysis of representative samples of the prepared diets indicated that there were only
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50 95 relatively small differences in dry matter, ash, protein and amino acid contents.
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Table 1 near here

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3 97 The dietary treatments were fed to 6 replicate pens of male broilers. Twenty four pens
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5 98 were used within the environmentally controlled house. The dietary treatments were randomly
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8 99 allocated to pens within positional blocks.
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12 101 **Broiler chicken management and feeding**

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15 102 The experiment was conducted under the guidance of the Research Ethics Committee of
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17 103 Harper Adams University College. A total of 1900 1-d-old male Ross 308 broilers was reared
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19
20 104 as a single flock in a solid-floored pen in an environmentally-controlled house. The birds
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22 105 were fed a proprietary, nutritionally-complete, broiler-starter diet that contained no antibiotic
23
24 106 or anticoccidial drugs for the first 16 d and provided with adequate feeders and drinkers for
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26
27 107 the age and the number of birds.
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29 108 At 16 d of age, the birds were weighed and 74 were randomly allocated to each of 24
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31 109 pens within an environmentally-controlled house, so that 1776 birds were used in the
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33
34 110 experimental design (24 pens x 74 birds). Each of the 24 pens had a solid concrete floor with
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36 111 an area of 1.5 m x 3 m covered with a bedding of 4 parts new wood shavings to one part
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38 112 reused litter material. The reused litter was from a previous flock reared on the same poultry
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40
41 113 unit. This flock had no clinical NE but some sub-clinical NE and sub-clinical coccidiosis
42
43 114 would have been expected.
44

45 115 For the following 16 d, the birds were fed the experimental diets. *Ad libitum* feed and
46
47 116 water were provided during the experimental period. Feed was provided in hanging tube
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50 117 feeders and the levels were managed to minimise wastage. House temperatures and humidity
51
52 118 were controlled to provide optimum growing conditions for the age of bird using the current
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54 119 (online) Management Guide for Ross 308 broilers (Aviagen, 2009). Light was provided for 23
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57 120 h/d. Over the experimental period, the growth and feed intakes of the birds were recorded. In
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60 121 addition, the birds in each pen were inspected daily and mortalities recorded.

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5 123 **Data collection**6
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8 124 *Lesion scoring*9
10 125 Randomly-selected birds were sampled from each of the 24 pens on d 5 and at the end of the
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12 126 feeding period (d 12, 13, 14 and 15). On d 5, 4 birds from each pen were killed by cervical13
14 127 dislocation and 5 ml of blood collected from the jugular vein and transferred into glass tubes,15
16 128 and ileal contents were collected and pooled for each pen. At 12, 13, 14 and 15 d, 8 birds17
18 129 from each replicate pen (two birds per day) were randomly selected, killed by cervical19
20 130 dislocation and 5 ml of blood collected from the jugular vein and transferred into glass tubes.21
22 131 The intestinal tract and liver were removed and the ileal contents collected from individual23
24 132 birds. Each liver was inspected for the lesions of hepatitis or cholangiohepatitis which are25
26 133 consistent with the pathological changes of NE (Lovland and Kaldhusdal, 1999; Sasaki *et al.*,27
28 134 2000). Four 8-cm intestinal sections were taken; the first of these sections was the duodenal29
30 135 loop, the second was proximal jejunum beginning immediately after the distal end of the31
32 136 duodenal loop, the third was either side of Meckel's diverticulum (mid small intestine) and33
34 137 the fourth terminated at the ileo-caecal junction (distal ileum). All 4 sections were35
36 138 immediately incised, washed in normal saline and the mucosal surfaces were inspected and37
38 139 any necrotic lesions recorded. A scoring system originally established by Truscott and Al-39
40 140 Sheikhly (1977), but modified by Gholamiandehkordi *et al.* (2007), was further slightly41
42 141 modified to score intestinal lesions. The scoring system was: score 0: no lesions, score 1:43
44 142 focal necroses (1-10 mm diameter), score 2: necrotic patches (1-2 cm diameter), score 3:45
46 143 coalesced necroses and score 4: pseudo-membrane covering the whole epithelial surface. The47
48 144 mucosal surfaces of each of the 4 gut wall samples were scored for necrotic lesions.49
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60 146 *Enumeration of Clostridium perfringens and coccidial oocysts*

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3 147 The duodenal sections were used for the quantification of *C. perfringens* and coccidial
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5 148 oocysts. Each duodenal sample was washed 4 times in sterile Phosphate Buffered Saline
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8 149 (PBS) at pH 7.2, weighed, added to the same weight of dilution buffer (Bio-X Diagnostics,
9
10 150 Jemelle, Belgium), stomachised in a sterile stomacher bag and the resulting liquid frozen at -
11
12 151 20°C. Duodenal extracts were subsequently thawed at room temperature and vortexed for the
13
14
15 152 quantification of coccidial oocysts. The extracts were diluted (x 6) with saturated NaCl,
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17 153 placed into both sides of a McMaster counting chamber and allowed to settle for 5 min. The
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19 154 number of coccidial oocysts (all species found in the sample) was counted at 10-times
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21 155 magnification.

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24 156 *C. perfringens* in the caecal contents and in the duodenal mucosal sample were
25
26 157 quantified with a double antibody sandwich ELISA (McCourt *et al.*, 2005), using the BIO K
27
28 158 086 – *Clostridium perfringens* antigen detection kit (Bio-X Diagnostics, Jemelle, Belgium).
29
30 159 Caecal contents were collected in a sterile container and diluted (x2) with dilution buffer
31
32 160 (Bio-X Diagnostics, Jemelle, Belgium), vortexed and frozen at -20°C before analysis. Both
33
34 161 duodenal extracts and diluted caecal contents were thawed and vortexed before being allowed
35
36 162 to settle for 10 min for the enumeration of *C. perfringens*. Standardisation of the ELISA was
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38 163 done as described previously (Palliyeguru *et al.*, 2010).
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44 165 *Quantification of serum α -toxin antibodies*

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46 166 The blood collected from each bird was allowed to clot for 8-10 h at room temperature. The
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48 167 serum was separated from the coagulum and stored at -20°C. Twelve serum samples from
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50 168 each pen were compared for antibody levels developed against the alpha toxin of *C.*
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52 169 *perfringens* using an indirect ELISA according to the method described by Heier *et al.* (2001)
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54 170 and Lovland *et al.* (2003) with slight modifications as described below. Each well of the
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56 171 Nunc-immunoplates (F96 Maxisorp, 735-0083, Thermo Fisher Scientific, Rochester, NY)

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3 172 was coated with antigen, phospholipase C type XIV (Sigma-Aldrich P 4039, St. Louis, MO)
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5 173 10 µg/ml in sodium carbonate and bicarbonate coating buffer (pH 9.6) by incubating the 100
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7
8 174 µl of antigen in each well overnight at 4°C. The plates were washed three times with PBS at
9
10 175 pH 7.2 with 0.05% Tween 20 (PBST). A volume of 150 µl of 1% bovine serum albumin in
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12 176 PBST was added into each well as a blocking buffer and incubated for 2 h. The plates were
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14
15 177 washed again three times with PBST.

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18 178 Serum samples were diluted 1:250 in PBST and 50 µl of each sample was added into
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20 179 duplicate wells. After leaving the plates at 4°C overnight, the contents were washed with
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22 180 PBST. Rabbit anti-chicken immunoglobulin IgY (IgG) whole molecule conjugated with
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25 181 alkaline phosphatase (Sigma-Aldrich A9171, St. Louis, MO) was diluted (10^{-4}) in blocking
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27 182 buffer and 50 µl of diluted anti-chicken antibodies were added to each well and the plates
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30 183 incubated at 37°C. After 2 h, the plates were washed with PBST and tapped on absorbent
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32 184 paper to remove all droplets.

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35 185 The plates were incubated with 150 µl of para-nitrophenyl phosphate (Sigma-Aldrich P
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37 186 7998, St. Louis, MO) for 1 h at 37°C, and the reaction stopped with 50 µl of 2M NaOH. The
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39 187 optical density (OD) was determined at 405 nm using a microplate reader (Bench Mark 170-
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41 188 6850, Bio-Rad, Hercules, CA). Resulting OD values were pooled for each pen. The indirect
42
43 189 ELISA was not standardised using chicken α -toxin antibodies, so the pooled OD values were
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45 190 compared instead of the antibody titres.

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52 192 *Feed and digesta analyses*
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54 193 The experimental feed samples were oven dried to a constant weight at 105°C to determine
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56 194 the dry matter content. Nitrogen (N) was determined with an automatic analyser (LECO FP-
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58 195 528 N; LECO Corp. St Joseph, MI) by AOAC 968.06 (Dumas method) using EDTA as the
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60 196 standard (AOAC International, 2000). TIA was determined by measuring the inhibition of mg

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3 197 of bovine trypsin per 1 g of sample using the method described by Smith *et al.* (1980).
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5 198 Dietary amino acid samples were determined by ion exchange chromatography (Biochrom 20
6
7
8 199 analyser, Amersham Pharmacia Biotech, Pittsburgh, PA) with post-column ninhydrin reaction
9
10 200 as previously described (Palliyeguru *et al.*, 2010).

11
12 201 Ash and acid insoluble ash contents in feed and digesta samples were measured using a
13
14 202 modified method described by Atkinson *et al.* (1984). Approximately 5 g of diet (dry) or 1.0 g
15
16 203 of digesta (dry) was placed into a previously-weighed crucible and ashed at 610°C for 13 h.
17
18 204 The contents of the cooled crucibles were weighed and washed into a glass tube with 50 ml of
19
20 205 4M HCl. The tubes were boiled at 110°C for 30 min. The cooled contents were filtered and
21
22 206 washed with de-ionised water through an ash-less filter paper and the filter paper was again
23
24 207 ashed in the same crucible at 610°C for 13 h. Subsequently the crucibles were cooled in a
25
26 208 desiccator and weighed.

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28
29 209 Crude protein content in each feed sample was measured in 6 replicate samples and the
30
31 210 crude protein content in ileal digesta and the acid insoluble ash (indigestible marker) contents
32
33 211 in both feed and ileal digesta were measured in duplicate samples and the protein digestibility
34
35 212 coefficient of the ileal digesta calculated.

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38 214 **Statistical analysis**

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41 215 The variables of growth performance, necrotic lesion scores in the intestinal sections,
42
43 216 proportion of birds that had liver and gall bladder lesions, *C. perfringens* in the caecal
44
45 217 contents and duodenal mucosa, serum antibody levels for alpha toxin and the number of
46
47 218 coccidial oocysts on the duodenal mucosa were compared using a randomised block analysis
48
49 219 of variance. The 24 pens were used as the experimental units and individual bird or sample
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51 220 data from within a pen were pooled before statistical analysis. The treatment sums of squares
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53 221 of the three soya bean diets were partitioned using a polynomial contrast with the determined

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3 222 TIA in dietary treatments as the explanatory variate using *Genstat 10* (Lawes Agricultural
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5 223 Trust, 2007)

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RESULTS

11
12 226 There were no major differences in the determined nutrient compositions of the three soya
13 227 bean diets (Table 1). The determined TIA of the non-toasted full fat soya bean was 27.62
14
15 228 mg/g. The TIA of the treatment diets increased with increasing inclusion rate of non-toasted
16
17 229 soya bean (0, 100 and 200 g/kg). Determined TIA with the three increasing non-toasted soya
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19 230 bean diets and the potato protein diet were 1.90, 6.21, 8.46 and 3.72 mg/g respectively.
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21 231 Although increasing the content of non-toasted full-fat soya bean significantly increased feed
22
23 232 intake, there was a marked reduction ($P < 0.001$) in protein digestibility, weight gain and feed
24
25 233 conversion efficiency. Mortality was not significantly affected (Table 2). Tables 2 & 3 near here

26
27 234 There was a linear increase ($P < 0.001$) of sub-clinical NE lesions in the duodenum,
28
29 235 jejunum, mid small intestine and ileum with increasing dietary non-toasted soya bean (Table
30
31 236 3). There was a significant increase in the number of liver lesions in the birds fed all other
32
33 237 dietary treatments compared with the toasted soya bean diet. Caecal *C. perfringens* counts
34
35 238 increased with the increasing dietary non-toasted soya bean. Serum α -toxin antibodies were
36
37 239 higher ($P < 0.001$), on d 5 in the birds fed the 200 g/kg non-toasted soya bean diet compared
38
39 240 with the other two soya bean diets, although there were no significant treatment differences at
40
41 241 the end of the feeding period. Dietary treatment had no significant effect on the number of *C.*
42
43 242 *perfringens* colonised on the duodenal mucosa. Coccidial oocyst counts decreased ($P < 0.01$)
44
45 243 with increasing dietary non-toasted soya bean (Table 3).

46
47 244 There was a strong negative correlation ($P < 0.001$) between the necrotic lesion scores
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49 245 of the intestine and the growth and protein digestibility coefficient of the broilers.

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DISCUSSION

247
248 Although the toasting may have had other effects on the chemical composition of the full fat
249 soya bean, the high levels of TIA were probably the major cause of the reduced protein
250 digestibility in the non-toasted soya bean diets; Perilla *et al.* (1997) concluded that TIA gave
251 the best prediction of the feeding value of soya beans. The growth performance responses of
252 the broilers to the dietary treatments in the present experiment were expected, but the size of
253 the reduction in growth was relatively large in comparison with the results of Perilla *et al.*
254 (1997), who observed a 6.5% increase in feed intake and a 4.9% decrease in growth rates for
255 an increase in dietary TIA from 0 to 9.4 mg/g. In the present experiment, there was a 4.3%
256 increase in feed intake but a 22% decrease in growth rate from the low to high TIA diets.

257 When the non-toasted soya bean was fed to the broilers, the effect of trypsin inhibitors
258 on protein digestibility did not fully explain the reduction in growth performance, as reported
259 by Liener and Kakade (1980). Dietary antibiotics have beneficial effects on the growth
260 performances of birds fed non-toasted soya bean diets (Hill *et al.*, 1957), and there is a lack of
261 growth depression in germ-free birds fed dietary trypsin inhibitors (Coates *et al.*, 1970;
262 Hewitt and Coates, 1973); all these experiments indicated that microbial activity in the
263 intestine may exacerbate the negative effects on growth performances caused by poor protein
264 digestion. However, in the present experiment, the broilers were reared in conditions that
265 predisposed sub-clinical NE and it is possible that the increased severity of sub-clinical NE in
266 these dietary treatments further contributed to the poor growth rate and feed conversion
267 efficiency.

268 The results (Table 3) are clear evidence that dietary non-toasted soya bean produced
269 an increased incidence and severity of sub-clinical NE, although there was no significant
270 increase in the *C. perfringens* numbers in the duodenum. Proliferation of this causative
271 organism can be restricted to localised sites in the small intestine, so it is difficult to

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2
3 272 consistently demonstrate a difference in the numbers of *C. perfringens* by sampling a
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5 273 representative amount of mucosal surface. Conversely, there was a good correlation between
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8 274 the numbers of caecal *C. perfringens* and the intestinal necrotic lesion scores in the present
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10 275 experiment, as has been reported in other studies (Kaldhusdal *et al.*, 1999; Elwinger *et al.*,
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12 276 1992). Although the serum antibody levels for *C. perfringens* alpha toxin were increased
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15 277 following 5 d of feeding 200 g/kg non-toasted soya bean, the birds fed this diet had the lowest
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17 278 antibody levels at the end of the experimental period (Table 3). Malnutrition generally has
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19 279 negative effects on antibody production in farm animals (Lawrence and Fowler, 2002), and,
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21 280 according to Klasing (2007), nutritional deficiencies can seriously reduce the immune
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23 281 responses of chickens. In the present experiment, the birds fed the highest (200 g/kg) rate of
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25 282 non-toasted soya bean had the greatest damage in their intestinal mucosa. This may have
26
27 283 caused poorer nutrient absorption and impeded the humoral immune responses. In addition to
28
29 284 poor nutrition, damage to the gut-associated lymphoid tissues could have contributed to
30
31 285 depressed immune responses. Chickens do not have peripheral lymph nodes similar to those
32
33 286 of mammals, therefore gut-associated lymphoid tissues are important for protection against
34
35 287 intestinal pathogens (Sklan, 2004). B lymphocytes in the lamina propria initiate the humoral
36
37 288 antibody responses to pathogens that enter into the body through the gut wall, so the damage
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39 289 to the intestinal mucosa could have greatly suppressed gut-associated immune mechanisms
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41 290 (Sklan, 2004; van Dijk *et al.*, 2002) and contributed to the lower antibody levels in the 200-
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43 291 g/kg non-toasted soya bean treatment. Controversially, increased gut damage could have
44
45 292 improved the immune response allowing more systemic access to toxin. In such an instance, it
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47 293 is possible that the antibody response in this treatment group could have increased, come to an
48
49 294 earlier peak and then declined between the two sampling days.

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53 295 The factors in non-toasted soya bean that predispose the proliferation of *C.*
54
55 296 *perfringens* or influence their toxins are not clear. However, the causative agent of NE, *C.*

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3 297 *perfringens*, is a commensal bacterium of chicken intestines (Ewing and Cole, 1994). When
4
5 298 protein digestibility is low, the ileal contents will have a relatively high content of protein.
6
7
8 299 The growth of *C. perfringens* under *in vitro* conditions needs 11 amino acids (Sebald and
9
10 300 Costilow 1975). *C. perfringens* that are producing α -toxin need specific amino acids and
11
12 301 peptides for their proliferation (Nakamura *et al.*, 1968), so it is possible that the low protein
13
14 302 digestibility in the small intestine directly predisposed the more rapid multiplication of *C.*
15
16 303 *perfringens*. There is also a possibility that trypsin inhibitors may stabilise the *C. perfringens*
17
18 304 toxins and so increase the severity of their toxigenic effects.
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21

22 305 Soya beans contain lectins that are known anti-nutritive factors (Probert, 2004) which
23
24 306 have the ability to damage the intestinal epithelial cells (van Dijk *et al.*, 2002) and also
25
26 307 possibly mediate the adhesion of pathogenic bacteria on to the epithelial cell membrane.
27
28 308 Lectin concentration can be 10-20 mg/g of non-toasted soya bean (Clarke and Wiseman,
29
30 309 2000). However, the lectins are heat labile and Armour *et al.* (1998) and Probert (2004) found
31
32 310 that lectins in soya bean denature more readily than trypsin inhibitors.
33
34
35

36 311 A negative correlation of coccidial oocysts counts in the duodenal mucosa with the
37
38 312 severity of lesion scores was found in this experiment and also in an earlier experiment
39
40 313 (Palliyeguru *et al.*, 2010). This may be due to their exclusion from the proliferation sites when
41
42 314 *C. perfringens* invade the damaged intestinal epithelium (Williams *et al.*, 2003). It is therefore
43
44 315 possible that the coccidial oocysts in the mucosa of the small intestine reduced with an
45
46 316 increasing severity of NE, even though the initial coccidial damage in the mucosal epithelium
47
48 317 may have predisposed (Al-Sheikhly and Al-Saieg, 1980; Shane *et al.*, 1985) and aggravated
49
50 318 (Park *et al.*, 2008) the *C. perfringens* damage.
51
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53

54 319 The birds fed the potato protein diet had significantly poorer growth performance
55
56 320 (weight gain, feed intake and gain:feed) than the birds fed the soya bean diet with the lowest
57
58 321 TIA. The non-significant trend of lower protein digestibility of the potato protein diet was
59
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2
3 322 consistent with the determined TIA of this diet, even though it was not as low as the two soya
4
5 323 bean diets that contained some non-toasted soya bean and had much higher trypsin inhibitor
6
7 324 activities. The relatively low TIA in the potato protein diet was not expected, despite the
8
9 325 trypsin inhibitor activities of different samples of potato protein concentrate being known to
10
11 326 vary with the variety (Jadhav and Kadam, 1998) and the method of processing (Knorr, 1980,
12
13 327 1982). There were no significant differences in caecal *C. perfringens* counts and serum α -
14
15 328 toxin antibody concentrations between the potato protein and the soya bean diet with the
16
17 329 lowest TIA. This was consistent with the relatively small difference between these two diets
18
19 330 in protein digestibility and TIA. However, the birds fed the potato protein diet had a
20
21 331 significantly higher incidence of liver lesions, increased severity of duodenal lesions and a
22
23 332 lower feed intake compared with the low trypsin inhibitor soya bean diet. The heat-resistant
24
25 333 glycoalkaloids (Jadhav and Kadam, 1998) that are also contained within potato protein (Lokra
26
27 334 *et al.*, 2008) may have contributed to the severity of the disease in the potato protein fed birds.

28
29 335 The results of this experiment have demonstrated that variation in the amount of non-
30
31 336 toasted dietary soya bean not only affects growth performance of broilers but also affects the
32
33 337 incidence of sub-clinical NE in the flock. Although the maximum recommended TIA of soya
34
35 338 bean is 4 mg/g, there is considerable variation in activity below this level in commercially-
36
37 339 available samples (Probert, 2004). Many countries have now adopted a complete ban on the
38
39 340 use of antimicrobial growth promoters and so there is potential for an increase in the
40
41 341 incidence of sub-clinical NE in their commercial broiler flocks. Ensuring the lowest possible
42
43 342 TIA in soya bean samples would appear to be a valuable tool for improving bird health and
44
45 343 welfare and reducing the risk of serious financial losses from NE.

344

345

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Table 1. *Feed ingredients, calculated and determined chemical compositions of experimental diets containing 0, 100 or 200 g/kg non-toasted soya bean or potato protein*

Ingredients (g/kg)	Experimental diet supplementation			
	Non-toasted soya bean protein (g/kg)			Potato protein
	0	100	200	
Maize	504.7	504.7	504.7	696.0
Rapeseed meal	073.0	073.0	073.0	073.0
Dehulled soya bean meal	142.3	142.3	142.3	-
Full fat soya bean	200.0	100.0	-	-
Ground raw soya bean	-	100.0	200.0	-
Potato protein concentrate	-	-	-	163.1
Soya bean oil	23.25	23.25	23.25	12
Lysine HCl	2.75	2.75	2.75	1
Methionine	2.5	2.5	2.5	1.3
Threonine	2.5	2.5	2.5	-
Tryptophan	0.1	0.1	0.1	-
Limestone	12.6	12.6	12.6	11.4
Di Calcium Phosphate	8.8	8.8	8.8	13.0
Salt	5.0	5.0	5.0	5.0
Choline chloride	-	-	-	1.7
Vitamin and trace element premix ¹	2.5	2.5	2.5	2.5
Celite	20.0	20.0	20.0	20.0
<i>Calculated chemical composition²</i>				
Metabolizable energy (MJ/kg)	13.1	13.1	13.1	13.1
Calcium	8.9	8.9	8.9	8.9
Phosphorus	5.7	5.7	5.7	5.7
Sodium	2.1	2.1	2.1	2.1
<i>Determined chemical composition</i>				
Dry matter	928.1	925.2	916.3	927.8
Ash	50.0	49.6	49.2	57.8
Crude protein	226.5	226.6	229.5	216.0
Alanine	9.8	9.9	9.8	10.8
Arginine	12.9	12.7	12.8	10.0
Aspartic acid	19.9	19.7	19.9	20.5
Cystine	3.4	3.4	3.4	3.2
Glutamic acid	35.2	35.3	35.2	27.4
Glycine	8.6	8.7	8.7	9.3
Histidine	5.4	5.4	5.4	4.9
Isoleucine	8.5	8.1	8.5	9.1
Leucine	16.7	16.5	16.7	19.8
Lysine	13.1	13.1	13.1	13.3
Methionine	5.3	5.3	5.4	5.3
Phenylalanine	10.0	9.8	9.8	11.2
Proline	11.9	11.6	11.4	11.9
Serine	10.2	10.4	10.2	10.6
Threonine	10.3	10.5	10.4	10.4
Tyrosine	7.3	7.1	7.3	9.0
Valine	9.7	9.1	9.9	11.4

¹Vitamin-trace mineral premix for broilers (Target Feeds Ltd., Whitchurch, UK) added per kg: 800 mg Retinol, 150 mg Cholecalciferol, 1.3 g Tocopherol, 150 mg Thiamin, 500 mg

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3 Riboflavin, 150 mg Pyrodoxine, 750 mg Cyanocobalamin, 3 g Nicotinamide, 0.5 g
4 Pantothenic acid, 75 mg Folic acid, 6.25 g Biotin, 12.5 g Choline chloride, 1 g Iron, 50 mg
5 Cobalt, 5 g Manganese, 0.5 g Copper, 4 g Zinc, 50 mg Iodine, 10 mg Selenium, and 25 mg
6 Molybdenum
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8 ²Chemical composition was calculated with UK derived nutrient composition tables (Premier
9 Nutrition, 2008)
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Table 2. *Growth performance¹ and protein digestibility² in male broiler chickens fed nutritionally complete diets with different protein concentrates for 16 d (16-32 d of age)*

	Soya bean diets			Potato protein diet	SED	Probability of differences	
	Level of un-heated soya bean (g/kg)					Treatment differences	Linear effect of non-toasted soya bean
	0	100	200				
	Trypsin inhibitor activity mg/g (TIA)			(TIA 3.72)			
	1.90	6.21	8.46				
Weight gain (kg)	1.290	1.179	0.995	1.142	0.0208	< 0.001	< 0.001
Feed intake (kg)	1.887	1.936	1.969	1.764	0.0342	< 0.001	< 0.05
Gain:feed	0.684	0.609	0.506	0.647	0.0115	< 0.001	< 0.001
Mortality %	1.6	1.8	1.6	5.0	1.56	NS	NS
Protein digestibility coefficient	0.653	0.561	0.544	0.614	0.0204	< 0.001	< 0.001

¹Data are means of 6 pens of 74 broilers per pen.

²Data are means of 6 replicate pens of 12 sampled broilers per each pen.

NS=not significant.

Table 3. Necrotic lesion scores, Clostridium perfringens counts, coccidial oocyst counts and serum antibody levels for Clostridium perfringens alpha-toxin¹ in male broiler chickens fed nutritionally complete diets with different protein concentrates for 16 d (16-32 d of age)

	Soya bean diets			Potato protein diet	SED	Probability of differences		
	Level of un-heated soya bean (g/kg)					Trypsin inhibitor activity mg/g (TIA)	Treatment differences	Linear effect of non-toasted soya bean
	0	100	200					
				(TIA 3.72)				
<i>Necrotic lesion scores</i>								
Duodenum	2.38	2.92	3.19	2.63	0.078	< 0.001	< 0.001	
Jejunum	2.13	2.44	2.58	2.27	0.085	< 0.001	< 0.001	
Mid small intestine	2.02	2.19	2.25	1.97	0.064	< 0.01	< 0.01	
Ileum	1.65	1.88	2.02	1.71	0.096	< 0.05	< 0.01	
Proportion of birds with liver or gall bladder lesions	0.69	0.85	0.88	0.88	0.062	< 0.05	< 0.01	
<i>C. perfringens counts (log₁₀cfu/g)</i>								
Caecum	7.82	7.99	8.32	7.91	0.138	< 0.01	< 0.01	
Duodenum	7.53	7.71	7.60	7.62	0.152	NS	NS	
<i>Serum α-toxin antibody (OD₄₀₅) in the feeding period</i>								
Intermediate (d 5) ^{2,3}	0.357	0.354	0.509	0.340	0.0370	< 0.01	< 0.001	
End (d 12-15) ⁴	0.554	0.584	0.509	0.582	0.0691	NS	NS	
<i>Coccidial oocyst counts in duodenal mucosa (n/g)²</i>								
	4564	3193	2530	3188	456.7	< 0.01	< 0.001	

¹Data are means of 6 replicate pens with 8 broilers sampled from each pen.

²Data are means of 6 replicate pens with 4 broilers sampled from each pen.

³Birds were sampled on d 5 of the feeding period (20 d of age)

⁴Birds were sampled for 4 consecutive days; from d 12-15 of the feeding period (28-31 d of age).