

Immune modulation, growth performance, and nutrient retention in broiler chickens fed a blend of phytogetic feed additives

by Pirgozliev, V., Mansbridge, S.C., Rose, S.P., Lillehoj, H.S. and Bravo, D.

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DOI: <https://doi.org/10.3382/ps/pey472>



Pirgozliev, V., Mansbridge, S.C., Rose, S.P., Lillehoj, H.S. and Bravo, D. 2018. Immune modulation, growth performance and nutrient retention in broiler chickens fed a blend of phytogetic feed additives. *Poultry Science*.

16 October 2018

1 **Immune modulation, growth performance and nutrient retention in broiler chickens fed**
2 **a blend of phytogenic feed additives**

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4 V. Pirgozliev^{*,†}, S.C. Mansbridge[†], S.P. Rose[†], H.S. Lillehoj[‡], D. Bravo[§]

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6 ^{*}Avian Science Research Centre, Scottish Agricultural College, Auchincruive, Ayr, UK, KA6

7 5HW.

8 [†]The National Institute of Poultry Husbandry, Harper Adams University, Shropshire, UK,

9 TF10 8NB.

10 [‡]Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural
11 Research, Service-U.S. Department of Agriculture, Beltsville, MD 20705.

12 [§]Pancosma S.A., CH-1218, Geneva, Switzerland.

13

14 Corresponding author: vpirgozliev@harper-adams.ac.uk

15

16 V. Pirgozliev

17 The National Institute of Poultry Husbandry

18 Harper Adams University

19 TF10 8NB, Newport, Shropshire, UK

20 Phone +44 (0) 1952 820280

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23 Abbreviated title: Phytogenic feed additives for broilers

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25

26 **ABSTRACT**

27 This study aimed to assess the effect of a commercial blend of phytogetic feed additives (PA),
28 comprising 5 % carvacrol, 3 % cinnamaldehyde, and 2 % capsicum oleoresin on the modulation
29 of innate immune **biomarkers** of broiler chickens, their growth performance, dietary energy and
30 nutrient **retention**. Four-hundred day-old birds were assigned to one of four dietary treatments.
31 Two control diets based on either wheat (WC) or maize (MC) were each given with and without
32 PA at 100 g/t. Growth performance variables including feed intake (FI), weight gain (WG) and
33 feed conversion ratio (FCR) were recorded. Dietary N-corrected apparent metabolizable energy
34 (MEn), dry matter (DMR), nitrogen (NR) and fat **retention (FR) coefficients** were also
35 determined. Gene expression of **immune biomarkers (cytokines)** were determined in caecal
36 tonsil tissue from 21d old birds. Expression of IL2, IL18, IL10 and IL17C in the caecal tonsils
37 were upregulated ($P < 0.05$) in the birds fed maize based diets compared to the wheat fed birds.
38 Feeding PA supplemented diets downregulated the expression of CD40LG ($P < 0.001$), IFNG
39 and IL6 ($P < 0.05$). There was a cereal type x PA interaction ($P < 0.05$), as expression of IFNB
40 was downregulated in the birds fed PA supplemented MC but not WC. However, expression
41 of IL12B was downregulated in birds fed PA supplemented WC but there was no significant
42 ($P > 0.05$) change in expression levels in birds fed MC diets. Feeding MC diets gave greater
43 FI ($P < 0.001$) and ME ($P < 0.05$), but lower FCR ($P < 0.05$) compared to birds fed WC diets.
44 The WG and nutrient retention coefficients were not affected ($P > 0.05$) by cereal type.
45 Supplementary PA improved FI ($P < 0.05$), WG ($P < 0.001$), FCR ($P < 0.05$), MEn ($P < 0.05$),
46 MEn:GE ratio ($P < 0.05$) and FR ($P < 0.05$). In conclusion, dietary inclusion of PA improved
47 overall growth performance variables, energy and nutrient **retention** and intestinal cytokine
48 expression.

49

50 **Keywords:** plant extracts, broiler chickens, immune response, metabolizable energy

51 **INTRODUCTION**

52

53 Inclusion of phytogenic feed additives (PA) in diets aiming to improve performance and health
54 has been promoted for broiler chickens and other farm animals (Windisch et al., 2008; Wallace
55 et al., 2010). Supplementation of PA to broiler diets has been shown to improve growth
56 performance variables (Jamroz et al., 2003; Pirgozliev et al., 2015a,b), dietary available energy
57 and nutrient digestibility (Mountzouris et al., 2010; Bravo et al., 2011, 2014), as well as
58 improve innate immunity and host disease resistance (Lee et al., 2010, 2013), and antioxidative
59 status (Karadas et al., 2014). The efficiency of dietary PA may also be influenced by the
60 hygienic conditions where birds are reared (Pirgozliev et al., 2014). Addition of PA to animal
61 diets alters normal gut microflora in broiler chickens (Kim et al., 2015), decreasing the
62 prevalence of pathogens, preventing colonization of the gastrointestinal tract (Mitsch et al.,
63 2004; Oviedo-Rondón et al., 2006). There is also increasing evidence that through interactions
64 with the immune system, PA are capable of modulating immune responses (Kim et al., 2015).
65 In the absence of antibiotics to promote animal growth, dietary immunomodulation is a key
66 antibiotic alternative that can contribute to the enhancement of productivity and integrity of the
67 immune system in farm animals (Kumar et al., 2011; Munyaka et al., 2012). The present
68 experiment aimed to assess the effect of a commercial blend of PA, including carvacrol,
69 cinnamaldehyde and **capsicum oleoresin** on local expression of cytokine **biomarker** genes in
70 the caecal tonsils that may indicate modulation of the immune response of rapidly growing
71 broiler chickens. In addition, the growth performance, dietary N-corrected apparent
72 **metabolizable** energy (ME_n) and nutrient **retention** were measured to examine the effects of
73 the PA on production variables.

74

75 **MATERIALS AND METHODS**

76

77 The Animal Experimental Committee of the Scottish Agricultural College approved all
78 procedures.

79

80 ***Birds husbandry and experimental design***

81

82 Four-hundred male day-old Ross 308 chicks were obtained from a commercial hatchery
83 and were allocated to 40 floor pens, 10 birds in a pen. Each of the forty pens had a concrete
84 floor with an area of 2.1 m². All birds were placed on litter that was a mixture of approximately
85 10 % new (top-dressed) and 90 % used, obtained from a previous crop of broiler chickens
86 reared at the same site. The previously reared flock did not have any clinical health problems.
87 The birds were vaccinated for Infectious Bronchitis (IB) at the hatchery.

88 Birds were fed one of four diets. There were two control diets based on either wheat
89 (WC) or maize (MC) and were formulated to be nutritionally adequate but marginally lower
90 (about 5 %) than the optimum economic metabolizable energy (ME) concentration (Aviagen
91 Ltd, Edinburgh, UK) for male broilers between 0 and 21 days of age (Table 1). A further two
92 diets were prepared using the basal control diets supplemented with a commercial blend of PA
93 (XTRACT 6930; Pancosma S.A., Geneva, Switzerland) comprising 5 % carvacrol, 3 %
94 cinnamaldehyde, and 2 % capsicum oleoresin at 100 g/t. The PA was incorporated into the
95 diets in powder form. All the diets were offered in mash form. The diets did not contain any
96 coccidiostat, antimicrobial growth promoters or similar additives. Feed and water were offered
97 *ad libitum* to birds throughout the experiment.

98 Each diet was given to birds in 10 pens following randomization. The experimental
99 room was equipped with a positive pressure ventilation system to meet commercial
100 recommendations. During the study, the temperature was initially 33 °C and was gradually

101 reduced to 20 °C after birds were 20 d of age. The relative humidity was maintained between
102 50 % and 70 %. A standard lighting program for broilers was used, decreasing from 23:1 (hours
103 light:dark) from day old to 18h:6h at 7 days of age, which was maintained until the end of the
104 study. The feeding period ended when the birds were 21 days of age.

105 At twenty-one days of age, one bird from each pen was randomly selected, stunned and
106 killed by cervical dislocation and the left caecal tonsil was collected and stored in RNAlater[®]
107 (Sigma-Aldrich, USA) at – 80 °C prior to analysis on the relative expression of the genes of
108 interest (GOI) (Table 2).

109

110 ***Total RNA extraction and reverse transcription***

111

112 The analyses of relative expression of GOI in the caecal tonsils were performed by
113 qStandard (Middlesex, UK). Approximately 30 mg of macro-dissected caecal tonsil tissue per
114 sample (stored as previously described) was homogenized in 500 uL QIAzol lysis reagent for
115 10 minutes at 30 Hz in a TissueLyzer LT (Qiagen, UK). Lysates were mixed with 100 µL
116 chloroform, transferred to pegGold PhaseTrap tubes (PeqLab, UK) and centrifuged for 5
117 minutes in at room temperature. The aqueous phase was poured into fresh tubes, mixed with
118 1.5 volumes of ethanol and applied to Qiagen RNeasy columns (Qiagen, UK). RNA was
119 purified according to the manufacturer's instructions (Qiagen, UK). RNA integrity was
120 assessed using an Agilent Bionalyzer and RIN was > 8 for all samples. Purity and quantity
121 were measured using a NanoDrop spectrophotometer; for all samples the absorbance peak was
122 at 260 nm, A_{260/280} > 2 and A_{260/230} > 1. Eight-hundred ng of RNA were reverse
123 transcribed using a Quantitect reverse transcription kit (Qiagen, UK) in a 10 µL reaction
124 according to the manufacturer's instructions. This RT kit includes a mandatory gDNA wipe out
125 step. The completed reaction was diluted 10-fold with 5 µg/mL tRNA in water.

126

127 ***Quantitative Real-Time PCR***

128

129 Two microliters of cDNA were amplified in a 10 μ L reaction using Agilent Brilliant III
130 SYBR Ultra-Fast SYBR Green mix with each primer at a final concentration of 500 nmol/L.
131 The no-template control reaction contained 2 μ L of tRNA (0.5 μ g/mL). DNA standards (10^7 -
132 10^1 copies/rxn) for each gene were included in each run. Reactions were pipetted robotically
133 using a Qiagility (Qiagen, UK). Amplification parameters were: 95 $^{\circ}$ C for 3 minutes followed
134 by 40 cycles of 95 $^{\circ}$ C for 5 seconds, 57 $^{\circ}$ C for 1 second in a Rotor-Gene 6000. Melt curves
135 were checked for product specificity (single peak) and the presence of primer dimers. All
136 primers were designed to be intron-spanning so that any residual gDNA present could not be
137 detected and avoided known SNP and secondary structures. Assays (Table 2) were designed
138 by qStandard (www.qstandard.co.uk) and were tested for specificity by electrophoresis,
139 efficiency > 95 %, sensitivity to 10 copies/rxn, and linearity over 7 log by qPCR. Copy
140 numbers/reaction were derived from the standard curves using the Rotor-Gene software. The
141 four reference genes identified as the most stable using geNorm software (Vandesompele et
142 al., 2002) were B2M, GAPDH, PPIA and YWHAZ (Table 3). The normalization factor for
143 each sample was determined using the method of Vandesompele et al. (2002) to normalize GOI
144 copy numbers per reaction.

145

146 ***Analysis of feed and excreta***

147

148 Dry matter (DM) in feed and excreta was determined by drying of samples in a forced
149 draft oven at 105 $^{\circ}$ C to a constant weight (AOAC, 2005; method 930.15). Crude protein (6.25
150 \times N) in samples was determined by the combustion method (AOAC, 2005; method 968.06)

151 using a LECO FP-528 N (Leco Corp., St. Joseph, MI). Oil (as ether extract) was extracted with
152 diethyl ether by the ether extraction method (AOAC, 2005; method 954.02) using a Soxtec
153 system (Foss UK Ltd.). Gross energy (GE) values of samples were determined by an isoperibol
154 oxygen bomb calorimeter (model 6200; Parr Instrument Co., Moline, IL) using benzoic acid as
155 the reference material, following the manufacturer's recommendations.

156

157 ***Dietary MEn and nutrient retention***

158

159 At the end of the feeding period, two birds from each pen were randomly selected and
160 transferred to one of 40 wire-meshed metabolism pens (0.400 m² floor area) in a controlled
161 environment room. The same diets were fed to the birds as they received in the feeding period.
162 Feed and water were offered *ad libitum*. The birds were kept in the pens for 96 h, from 21 to
163 25 d age, and total excreta output was collected twice (every 48 h) from the trays beneath to
164 avoid fermentation losses. Spilled feed and feathers were removed, and excreta were collected
165 before weighing.

166 The coefficients for dry matter (DMR), nitrogen (NR) and fat retention (FR) were
167 calculated as the difference between the intake and the output of the respective nutrient and
168 this was divided by the intake of the nutrient. Dietary MEn was calculated as described by Hill
169 and Anderson (1958).

170

171 ***Statistical analysis of data***

172

173 Data were statistically analyzed by two-way ANOVA using a 2 x 2 factorial
174 randomized block arrangement of treatments. The main effects were the cereals (maize and
175 wheat) and additives (with and without PA) used. All data were analyzed with the ANOVA

176 procedure of GenStat 15 statistical software package (IACR, Rothamstead, Hertfordshire, UK).
177 In all instances, differences were reported as significant at $P < 0.05$.

178

179 RESULTS

180

181 No health problems were associated with use of cereal type or supplementary PA
182 throughout the experiment. Mortality was low ($< 3\%$) and not treatment associated.

183 The relative expression (as copy numbers per reaction) of cytokine GOI in the caecal
184 tonsils is presented in Table 4. Expression of IL2, IL18, IL10 and IL17C in the caecal tonsils
185 were upregulated ($P < 0.05$) in the birds fed the maize based diets. Dietary PA downregulated
186 the expression of CD40LG ($P < 0.001$), IFNG and IL6 ($P < 0.05$). There was a cereal type x
187 PA interaction ($P < 0.05$) for IFNB and IL12B (Table 4). Expression of IFNB was
188 downregulated in the birds fed PA supplemented maize based diets, although expression of
189 IL12B was downregulated in birds fed PA supplemented wheat based diet. The expression of
190 LITAF, TGFB1 and IL1B was not influenced ($P > 0.05$) by dietary treatments. The expression
191 of IL4 was undetectable for the majority of samples and therefore not included in the results
192 table.

193 Results of growth performance variables, dietary MEn, and nutrient retention
194 coefficients are presented in Table 5. Birds fed maize based diets had 14.2 % greater FI ($P <$
195 0.001), 4.6 % greater MEn ($P < 0.05$), but 10.7 % greater (less efficient) FCR ($P < 0.05$)
196 compared to birds fed wheat-based diets. The WG and nutrient retention coefficients were not
197 affected ($P > 0.05$) by cereals used in diet formulations.

198 Feeding PA increased FI by 6.1 % ($P < 0.05$), WG by 16.4 % ($P < 0.001$), dietary MEn
199 by 3.5 % ($P < 0.05$), MEn:GE ratio by 2.7 % ($P < 0.05$), dietary FD by 6.2 % ($P < 0.05$), and

200 reduced FCR (improved feed efficiency) by 9.4 % ($P < 0.05$). There were no dietary cereal x
201 PA interactions ($P > 0.05$) for any of the variables studied in Table 5.

202

203

DISCUSSION

204

205 Cytokines play a key role in both the adaptive and the innate immune system (Kim et
206 al., 2010; Lillehoj et al., 2011). It has been speculated that the benefit of using PA in animal
207 diets is associated with reduced intestinal inflammation resulting from a reduction of
208 proinflammatory cytokines. Chao et al. (2008) reported that cinnamaldehyde suppressed the
209 lipopolysaccharide-induced production of tumor necrosis factor (TNF), interleukin 6 (IL6) and
210 IL1, thus suggesting that the inclusion of cinnamaldehyde could show suppressive effects on
211 the production of various types of proinflammatory cytokines, which could explain the
212 mitigation of the severity of coccidiosis when cinnamaldehyde is included in feed (Lee et al.,
213 2010). Lee et al. (2013) also found that a mixture of capsicum and turmeric oleoresins was an
214 effective phytonutrient against clinical signs of experimental avian necrotic enteritis when
215 supplied in dietary form. Most cytokines have pleiotropic or redundant functions, and the level
216 of one cytokine is tightly regulated by other cytokines. The overall picture tends towards an
217 anti-inflammatory effect for the treatments supplemented with PA. The major proinflammatory
218 cytokines studied in this case were IFNG, IL6, IL18 and IL1b. As the expression of both IFNG
219 and IL6 cytokines were downregulated, this would indicate a lower inflammation level than in
220 the other groups, under normal conditions (since no pathogen challenge was given). The LITAF
221 is similar in that it regulates the expression of TNF-alpha (Hong et al., 2006). However, there
222 are other regulators of TNF so further investigation into TNF expression may be warranted,
223 particularly as there were differences in the IL6 and IFNG between the groups. If all the groups
224 were exposed to a bacterial challenge, it could explain why there are no differences in the

225 LITAF expression levels between groups – LITAF is particularly responsive to bacterial
226 products (LPS) so it would be much more difficult to observe differences in the expression of
227 this gene under the current experimental conditions.

228 The other two genes (CD40LG and IL12p40) are expressed mainly by activated macrophages
229 and dendritic cells, so these genes are a good indicator that an immune response is likely being
230 generated (perhaps in response to the microbial loading provided by the recycle litter). These
231 genes are also interesting in that they are involved in the activation of adaptive immunity
232 (activation of Th1 cells and B cells) and it might be worth investigating other aspects of the
233 adaptive immune response to check this.

234 The relatively low copy numbers of some GOI in this study, including IL4 (data not in
235 tables), INFB, IL2 and IL10, suggests the results should be interpreted with caution as
236 approaching the assay limit of detection may reduce the reliability of the data and limit the
237 ability to interpret the full profile of cytokine responses likely to be involved in a bacterial
238 challenge (Reid et al., 2016). The production of proinflammatory cytokines would be expected
239 in those macrophages where an inflammatory response occurs (Kaiser et al., 2000). In the
240 present study, the majority of the cytokines showed significantly modulated expression in
241 response to PA and/or diet type, thereby indicating their ability to modulate the innate immune
242 response in the caecal tonsil tissue cells. Kim et al. (2010) also found that the local production
243 of proinflammatory cytokines was significantly decreased when feeding the same mixture of
244 PA to chickens.

245 In mammals, IL1B and IL6 are both critical for activating the immune response and
246 synthesizing acute-phase proteins (Giansanti et al., 2006). It is speculated that these two
247 proinflammatory cytokines might be essential in the early phase of the inflammatory responses.
248 The individual components in the mixture of PA, particularly capsicum, have been shown to
249 have a protective function in the gastrointestinal mucosa increasing resistance to *Salmonella*

250 colonization and organ invasion in broilers (McElroy et al., 1994). Karadash et al. (2014)
251 reported an increase in hepatic antioxidants when the same PA blend was fed to broilers.
252 Dhuley (1999) showed that carvacrol and cinnamaldehyde increase the activity of the
253 antioxidant enzymes of the cells of the mucosa layer, which is known to be a protective system
254 for the tissue. The latter could be the basis for the mucosal (villus-regulated) protective effect
255 of PA previously observed by Jamroz et al. (2006). An improvement in health of the digestive
256 mucosa by PA has also been demonstrated in piglets (Manzanilla et al., 2004, 2006).

257 Lee et al. (2010), reported that a combination of different phytonutrients (including
258 capsaicin) promoted local protective immunity against avian coccidiosis caused by intestinal
259 protozoan parasites, *Eimeria* spp., confirmed by increased levels of serum antibodies and
260 increased levels of proinflammatory cytokine production in the duodenum. However, Lee et
261 al. (2010) purposely challenged the birds with *E. acervulina*, thus severely provoking their
262 protective immunity. In the present study, birds were placed on recycled litter only, and no
263 specific challenge was applied, thus the measured copy numbers per reaction were relatively
264 low. Rearing conditions influence the responses to phytogetic feed additives (Pirgozliev et al.,
265 2014), thus should be taken into account for a more complete interpretation of the experimental
266 data emanating from experiments involving PA.

267 The growth performance results observed in the present study confirmed the growth-
268 stimulating effect of the mixture of commercial PA that contains carvacrol, cinnamaldehyde,
269 and capsicum oleoresin, in agreement with previous reports (Jamroz et al., 2003; Bravo et al.,
270 2011). The relatively low weight of the birds compared to the breeders recommendations
271 (Aviagen Ltd., Edinburgh, UK), was likely due to the 5 % lower ME than commercial
272 recommendations and feeding a mash diet, rather than a pelleted diet. For example, Pirgozliev
273 et al. (2016) noted approximately 20 % lower body weight in birds fed mash diets, compared
274 to pellets.

275 Compared to maize, wheat contains more water-soluble NSP, a carbohydrate complex
276 with high water holding capacity, which may increase intestinal digesta viscosity, and reduce
277 feed intake (Annison et al., 1996). Producing isoenergetic diets required more oil in wheat-
278 based diets formulation, which explains the greater metabolizable energy of them compared to
279 the maize-based diets.

280 Feeding the mixture of phytonics improved dietary metabolizable energy in accord
281 with Mountzouris et al. (2010) and Bravo et al. (2011). As previously speculated (Bravo et al.,
282 2011), the metabolizable energy effect of PA may be mediated by a direct increase in dietary
283 energy availability, by a decrease in the energy required for the maintenance of the digestive
284 tract, or a combination of both. This is supported by the increase in MEn:GE ratio (i.e.
285 improved dietary energy utilization) in this study. Mitsch et al. (2004) also demonstrated that
286 the same blend of PA reduced *Clostridium perfringens* colonization and proliferation in the gut
287 of broilers, thus reducing the energy used by the bird to maintain the digestive tract ecosystem.
288 In addition, Pirgozliev et al. (2015b) observed reduced heat production from broilers fed the
289 same PA supplemented diets, speculating a reduced maintenance requirement of the birds.

290 In agreement with Hernández et al. (2004), fat retention was improved, further
291 supporting the positive PA effect on available energy and growth performance variables. The
292 reason for the enhanced availability with PA could be due to increased digestive secretions, as
293 reported for the effect of capsicum oleoresin on pancreatic and intestinal enzyme activity in
294 rats (Platel and Srinivasan, 2001), and on bile flow and bile acid secretion (Ganesh Bhat et al.,
295 1984). Moreover, mixtures of spices exhibited an additive effect regarding their pancreatic
296 enzyme stimulation compared with the spices taken individually (Platel et al., 2002).

297 The results show that supplementation of broiler chickens' diet with a standardized
298 combination of 5 % carvacrol, 3 % cinnamaldehyde and 2 % capsicum oleoresin have
299 beneficial effects on the performance as well as host innate immunity, possibly through

300 immune modulation (reduced inflammation) of local effector cells in the gut. Since dietary
301 phytochemicals influence gut microflora (Kim et al., 2015) in commercial broiler chickens,
302 future studies to investigate the role of dietary phytochemicals on gut microbiota and immune
303 system cross talks need to be carried out.

304 In conclusion, the present results indicate that a dietary combination of PA, including
305 carvacrol, cinnamaldehyde, and capsicum oleoresin, improved the nutritional value of a wheat-
306 and maize-based diets fed to young broiler chickens. A reduction in CD40LG and IFNG
307 indicate an anti-inflammatory effect of the PA mixture, suggesting an explanation for the
308 improvement in growth performance and MEn seen in the present study.

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Table 1. Ingredient composition of the experimental control diets (as-fed basis)

Ingredients, g/kg	MC	WC
Maize	528.6	-
Wheat	-	546.8
Soybean meal (48)	313.0	274.9
Vegetable oil	10.0	35.0
Barley	63.3	58.4
Rye	50.0	50.0
Dicalcium phosphate	14.3	14.3
Limestone	11.5	11.5
NaCl	3.3	2.7
Lysine HCL	1.5	1.5
Methionine	3.5	3.9
Vitamin mineral premix ¹	1.0	1.0
Total	1000	1000
Calculated analysis (as-fed basis)		
ME, MJ/kg	12.13	12.13
Crude protein, g/kg	215	215
Crude fat, g/kg	34	47
Calcium, g/kg	8.3	8.4
Non-phytate P, g/kg	4.4	4.5
Lysine, g/kg	12.3	12.3
Methionine + Cysteine, g/kg	9.5	9.5
Analyzed values (as fed basis)		
Dry matter, g/kg	864	872
Crude protein, g/kg	197	198
Crude fat, g/kg	35	47

470 ¹ The premix provided (units/kg diet): 12,000 IU retinol, 5,000 IU cholecalciferol, 34 mg α -
471 tocopherol, 3 mg menadione, 2 mg thiamine, 7 mg riboflavin, 5 mg pyridoxine, 15 μ g
472 cobalamin, 50 mg nicotinic acid, 15 mg pantothenic acid, 1 mg folic acid, 200 μ g biotin, 80 mg
473 Fe as iron sulfate (30 %), 10 mg Cu as a copper sulfate (25 %), 100 mg Mn as manganous
474 oxide (62 %), 80 mg Zn as zinc oxide (72 %), 1 mg I as calcium iodate (52 %), 0.2 mg Se as
475 sodium selenite (4.5 %), and 0.5 mg Mo as sodium molybdate (40 %).
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480 **Table 2.** RT-qPCR assays¹ for quantification of gene expression in *Gallus gallus* caecal tonsil tissue

Gene symbol	Gene	Accession number	Primer sequences (5'-3')	Product length, bp	Location
CD40LG	CD40 ligand	NM_204733	S – TAGGACAGCCAGTGAGGAGT A – TTCTCCTCTGCCACAGATGTC	99	S – Exon 3 A – Exons 4 & 5
LITAF	Lipopolysaccharide – induced TNF factor	NM_204267	S – CTGTTCTATGACCGCCAGT A – CTATGCACCCCAGCAGGAAGA	130	S – Exon 4 A – Exons 4 & 5
TGFB1	Transforming growth factor beta 1	NM_001318456	S – CTGTACCAGGGTTACGGCAA A – CCCATCTCACAGGGACAGTG	174	S – Exon unknown A – Exon unknown
IFNB	Interferon, beta 1, fibroblast	NM_001024836	S – CTCTTGCTTCTGCCAGCTCT A – CACGTCTTGTTGTGGGCAAG	141	S – Exon 1 A – Exon 1
IL1B	Interleukin 1, beta	NM_204524	S – CTCACAGTCCTTCGACATCTTC A – TCACTTTCTGGCTGGAGGAG	123	S – Exon 4 A – Exons 4 & 5
IFNG	Interferon, gamma	NM_205149	S – AAAGCCGCACATCAAACACA A – AGTCGTTTCATCGGGAGCTTG	116	S – Exon 3 A – Exons 3 & 4
IL4	Interleukin 4	NM_001007079	S – ATGACATCCAGGGAGAGGTTT A – TGCTCCACAATCCCTTTCTT	166	S – Exons 2 & 3 A – Exon 3
IL6	Interleukin 6 (interferon, beta 2)	NM_204628	S – CTTCGACGAGGAGAAATGCC A – TAGCACAGAGACTCGACGTT	120	S – Exons 2 & 3 A – Exon 3
IL2	Interleukin 2	NM_204153	S – ACCAACTGAGACCCAGGAGT A – CGGTGTGATTTAGACCCGTAAGA	170	S – Exons 2 & 3 A – Exons 3 & 4
IL12B	Interleukin 12B	NM_213571	S – ACTACTGTCCATTTGCCGAAG A – GGTCTGGCTTTATGATATCTCTGA	121	S – Exon 4 A – Exons 4 & 5
IL17C	Interleukin 17C	XM_003641945	S – AGCCTCACGAGAGATCCATC A – CCTCCCTGTCTTCACATCCAC	125	S – Exon 1 A – Exon 2
IL18	Interleukin 18	NM_204608	S – AGTTGCTTGTGGTTCGTCCA A – TCCACTGCCAGATTTACCTC	80	S – Exon 2 A – Exon 2 & 3
IL10	Interleukin 10	NM_001004414	S – GAGTTTAAGGGGACCTTTGGC A – CTCTGCTGATGACTGGTGCT	107	S – Exons 2 & 3 A – Exon 3

481 S = Sense primer; A = Anti-sense primer. ¹primer sequences are provided in the interest of transparency but remain the intellectual property
 482 of qStandard (www.qstandard.co.uk)

483

484 **Table 3.** RT-qPCR assays¹ for reference gene selection for **normalization** of gene expression in *Gallus gallus* caecal tonsil tissue

Gene symbol	Gene	Accession number	Primer sequences (5'-3')	Product length, bp	Location
ACTB	Actin, beta	NM_205518	S – TGACAATGGCTCCGGTATGTG A – CAACCATCACACCCTGATGTC	107	S – Exon 1 A – Exons 1 & 2
B2M	Beta - 2 microglobulin	NM_001001750	S – GTACTCCGACATGTCCTTCAAG A – CACAGCTCAGAACTCGGGAT	157	S – Exon 2 A – Exons 2 & 3
GAPDH	Glyceraldehyde - 3- phosphate dehydrogenase	NM_204305	S – TGTGACTTCAATGGTGACAGC A – CCAAATCATTGTCATACCAGGA	107	S – Exon 9 A – Exons 9 & 10
PPIA	Peptidylprolyl isomerase A (cyclophilin A)	NM_001166326	S – TGACAAGGTGCCATAACAG A – CGTAAAGTCACCACCCTGAC	127	S – Exon 1 A – Exons 2 & 3
YWHAZ	Tyrosine 3 – monooxygenase / tryptophan 5 – monooxygenase	NM_001031343	S – TCTTGATCCCCAATGCTTCG A – TGCTCCACAATCCCTTTCTT	122	S – Exon 2 A – Exons 2 & 3

485 S = Sense primer; A = Anti-sense primer.

486 ¹primer sequences are provided in the interest of transparency but remain the intellectual property of qStandard (www.qstandard.co.uk)

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492 **Table 4.** Relative expression (copies per reaction) of selected genes expressed in *Gallus gallus* caecal tonsil tissue¹

Item ²	Treatment factor ³											
	CD40LG	LITAF	TGFB1	IFNB	IL1B	IL12B	IFNG	IL2	IL18	IL6	IL10	IL17C
Diet												
W	230	15576	84	8	56	83	29	3	206	25	6	2
M	222	14064	99	12	47	75	24	9	289	24	18	6
PA												
-	282	15900	103	12	50	95	33	5	232	38	10	5
+	170	13740	80	9	53	63	20	7	263	11	14	3
SEM	14.8	1219.9	13.1	2.7	5.9	11.3	4.3	2.0	26.5	6.3	2.6	1.4
Diet and PA												
Wheat -	295	17483	82	6 ^a	54	122 ^a	39	2	227	39	4	1
Wheat +	166	13669	86	11 ^{ab}	59	44 ^b	19	4	186	11	9	2
Maize -	269	14318	125	18 ^b	46	69 ^b	27	8	237	37	17	9
Maize +	175	13811	73	6 ^a	48	82 ^{ab}	19	10	341	11	19	3
SEM	20.9	1725.2	18.5	3.8	8.4	15.9	6.0	2.8	37.5	8.9	3.6	2.0
Probabilities of statistical differences												
Diet	NS	NS	NS	NS	NS	NS	NS	0.037	0.039	NS	0.005	0.049
PA	<0.001	NS	NS	NS	NS	0.058	0.032	NS	NS	0.007	NS	NS
Diet x PA	NS	NS	NS	0.045	NS	0.009	NS	NS	0.068	NS	NS	0.070

493 ¹Based on the left caecal tonsil collected from 21d old birds and stored in RNAlater[®] (Sigma-Aldrich, USA) at – 80 °C prior analysis and
 494 10 observations per treatment.

495 ²W = wheat-based diet; M = maize-based diet; PA = supplemental phytogetic feed additive (100 g/t); (–) = diet was not supplemented with
 496 PA; (+) = diet was supplemented with PA.

497 ³CD40LG = CD40 ligand; LITAF = lipopolysaccharide-induced tumor necrosis factor-alpha factor; TGFB1 = transforming growth factor,
 498 beta 1; IFNB = interferon beta; IL1B = interleukin 1 beta; IL12B = interleukin 12, subunit beta; IFNG = interferon gamma; IL2 = interleukin
 499 2; IL18 = interleukin 18; IL6 = interleukin 6; IL10 = interleukin 10; IL17C = interleukin 17C.

500 **Table 5.** Broiler growth performance, dietary energy and nutrient retention¹

Item ²	Treatment factor ³							
	FI	WG	FCR	MEn	MEn:GE	DMR	NR	FR
Diet								
W	0.824	0.619	1.352	13.35	0.707	0.723	0.595	0.831
M	0.941	0.635	1.496	13.99	0.726	0.730	0.621	0.843
PA								
-	0.857	0.580	1.494	13.43	0.704	0.721	0.608	0.812
+	0.909	0.675	1.353	13.90	0.729	0.732	0.607	0.862
SEM	0.0114	0.0159	0.0329	0.144	0.0076	0.0091	0.0118	0.0141
Probabilities of statistical differences								
Diet	<0.001	NS	0.004	0.004	0.083	NS	NS	NS
PA	0.003	<0.001	0.005	0.031	0.031	NS	NS	0.019
Diet x PA	NS	NS	NS	NS	NS	NS	NS	NS

501 ¹Based on feeding period from 1 to 21 d of age for growth performance and from 21 to
 502 25 d of age for ME, ME:GE, total tract DM retention coefficient, total tract N retention
 503 coefficient and total tract fat retention coefficient and 10 observations per treatment.

504 ²W = wheat-based diet; M = maize-based diet; PA = supplemental phytogenic feed
 505 additive (100 g/t); (-) = diet was not supplemented with PA; (+) = diet was
 506 supplemented with PA.

507 ³FI = feed intake; WG = weight gain; FCR = feed conversion ratio; MEn = dietary
 508 apparent metabolizable energy corrected for N retention; GE = dietary gross energy;
 509 MEn:GE = metabolizability of dietary GE; DMR = total tract DM retention coefficient;
 510 NR = total tract N retention coefficient; FR = total tract fat retention coefficient.