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Original Research Article

Priming of intestinal cytoprotective genes and antioxidant capacity by dietary phytogenic inclusion in broilers

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ABSTRACT

The potential of a phytogenic premix (PP) based on ginger, lemon balm, oregano and thyme to stimulate the expression of cytoprotective genes at the broiler gut level was evaluated in this study. In particular, the effects of PP inclusion levels on a selection of genes related to host protection against oxidation (catalase [*CAT*], superoxide dismutase 1 [*SOD1*], glutathione peroxidase 2 [*GPX2*], heme oxygenase 1 [*HMOX1*], NAD(P)H quinone dehydrogenase 1 [*NQO1*], nuclear factor (erythroid-derived 2)-like 2 [*Nrf2*] and kelch like ECH associated protein 1 [*Keap1*]), stress (heat shock 70 kDa protein 2 [*HSP70*] and heat shock protein 90 alpha family class A member 1 [*HSP90*]) and inflammation (nuclear factor kappa B subunit 1 [*NF-κB1*], Toll-like receptor 2 family member B (*TLR2B*) and Toll-like receptor 4 [*TLR4*]) were profiled along the broiler intestine. In addition, broiler intestinal segments were assayed for their total antioxidant capacity (TAC). Depending on PP inclusion level (i.e. 0, 750, 1,000 and 2,000 mg/kg diet) in the basal diets, 1-d-old Cobb broiler chickens ($n = 500$) were assigned into the following 4 treatments: CON, PP-750, PP-1000 and PP-2000. Each treatment had 5 replicates of 25 chickens with ad libitum access to feed and water. Data were analyzed by ANOVA and means compared using Tukey's honest significant difference (HSD) test. Polynomial contrasts tested the linear and quadratic effect of PP inclusion levels. Inclusion of PP increased ($P \leq 0.05$) the expression of cytoprotective genes against oxidation, except *CAT*. In particular, the cytoprotective against oxidation genes were up-regulated primarily in the duodenum and the ceca and secondarily in the jejunum. Most of the genes were up-regulated in a quadratic manner with increasing PP inclusion level with the highest expression levels noted in treatments PP-750 and PP-1000 compared to CON. Similarly, intestinal TAC was higher in PP-1000 in the duodenum ($P = 0.011$) and the ceca ($P = 0.050$) compared to CON. Finally, increasing PP inclusion level resulted in linearly reduced ($P \leq 0.05$) expression of *NF-κB1*, *TLR4* and *HSP70*, the former in the duodenum and the latter 2 in the ceca. Overall, PP inclusion consistently up-regulated cytoprotective genes and down-regulated stress and inflammation related ones. The effect is dependent on PP inclusion level and the intestinal site. The potential of PP to beneficially prime bird cytoprotective responses merit further investigation under stress-challenge conditions.

1. Introduction

In intensive poultry farming, birds face an array of stressor challenges of endogenous (e.g. metabolic) and environmental (e.g. temperature, feed composition and xenobiotics) origin that if not controlled promptly and adequately will lead to oxidative stress. The latter may result in cellular protein oxidation and lipid peroxidation, DNA damage and inflammation with detrimental effects for poultry performance, health and product quality (Adly, 2010; Sahin et al., 2013; Lee et al., 2017; Da Silva et al., 2018; Carvalho et al., 2019).

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Diet beyond its mere contribution to direct antioxidant compounds such as vitamins, polyphenols, trace minerals, carotenoids and cofactors (Allen and Tresini, 2000; Adly, 2010; Lee et al., 2017), may contain bioactive compounds conferring an additional antioxidant effect by stimulating the innate detoxifying and antioxidant defense system. The latter involves the inducible gene expression of cytoprotective proteins with detoxifying, antioxidant and anti-inflammatory functions (Kohle and Bock, 2006; Wullaert et al., 2011; Huang et al., 2015). Contemporary knowledge suggests that phytogenic compounds derived from various vegetables, fruits, spices and herbs activate a signaling pathway known as the Kelch-like ECH-associated protein-1 (*Keap1*)/nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*)/antioxidant response element (*ARE*) and induce the expression of antioxidant and phase II detoxifying enzymes (Stefanson and Bakovic, 2014; Ahmed et al., 2017; Lee et al., 2017). Moreover, the *Keap1/Nrf2/ARE* pathway is tightly linked with the transcription factor nuclear factor kappa B subunit 1 (*NF-κB1*) pathway known to mediate immune responses to infections and inflammation, thereby actively affecting epithelial homeostasis (Wullaert et al., 2011; Stefanson and Bakovic, 2014; Wardyn et al., 2015).

So far, a limited number of studies have dealt with the *Keap1/Nrf2/ARE* pathway modulation in broilers. For example, the up-regulation of certain *ARE* genes by dietary phytogenics in the broiler jejunal and cecal tissues, was correlated with reduced oxidative stress (Mueller et al., 2012). Phytogenic inclusion in heat-stressed broilers alleviated stress through stimulation of the *Nrf2/ARE* pathway and up-regulation of antioxidant and detoxifying enzymes (Sahin et al., 2013; Liu et al., 2014; Song et al., 2017; Zhang et al., 2018). In addition, activation of the *Nrf2* pathway by dietary phytogenics such as curcumin and grape seed extract alleviated aflatoxin B1-induced immunotoxicity and oxidative stress in broilers (Wang et al., 2018; Rajput et al., 2019). However, it is clear the magnitude of antioxidant and anti-inflammatory capacity of phytogenics will largely depend on the chemical properties of phytogenic constituents, their inclusion level in the diet, the absorptive and post-absorptive phytogenic kinetics and host digestive physiology (Brenes and Roura, 2010; Mueller et al., 2012; Sahin et al., 2013; Stefanson and Bakovic, 2014; Paraskeuas et al., 2016, 2017; Lee et al., 2017; Huang and Lee, 2018).

Therefore, the potential of phytogenics to activate the *Keap1/Nrf2/ARE* pathway at intestinal level could be an additional asset in protecting bird performance and health that merits further investigation. We have recently shown that dietary inclusion of a phytogenic premix (PP) based on ginger, lemon balm, oregano and thyme in broilers increased carcass and meat yield, and in addition PP enhanced the capacity of meat and liver tissues to resist oxidation (Mountzouris et al., 2019). The aim of this work was to generate further knowledge for PP potential to modulate the expression of critical *Keap1/Nrf2/ARE* pathway genes along the broiler intestine. Moreover, the expression of other relevant genes for inflammation and stress such as *NF-κB1*, Toll like receptors (*TLR2* and *TLR4*) and HSP (*HSP70* and *HSP90*) was also profiled along the broiler intestine. Finally, in addition to the molecular markers assessed, the total antioxidant capacity (TAC) of each intestinal segment was determined as a biochemical indicator of the intestinal antioxidant status.

2. Materials and methods

2.1. Birds, housing and experimental treatments

This study continued the analytical approach initiated in previous research work (Mountzouris et al., 2019). Housing, management and care of the animals complied with the current European Union Directive on the protection of animals used for scientific purposes (EC 43/2007; EU 63/2010) and the experimental protocol was approved by the relevant national authority for animal experimentation.

Briefly, 500, as hatched, 1-d-old Cobb 500 broilers, vaccinated for Marek's disease, infectious bronchitis and Newcastle disease were obtained from a commercial hatchery. Upon arrival at the experimental facility, broilers were randomly allocated in 4 experimental treatments. Each treatment had 125 broilers arranged in 5 replicate floor pens of 25 broilers each. Depending on the inclusion level of the PP in the basal diet, the experimental treatments were: CON (basal diet with no PP addition); PP-750 (basal diet containing PP at 750 mg/kg diet); PP-1000 (basal diet containing PP at 1,000 mg/kg diet) and PP-2000 (basal diet containing PP at 2,000 mg/kg diet). Each treatment replicate was assigned to a clean floor pen (2 m^2) and birds were raised on rice hulls litter. All experimental treatments received a maize-soybean meal basal diet in mash form formulated to meet Cobb nutrient requirements according to a 3-phase feeding plan (e.g. starter [1 to 10 d], grower [11 to 22 d] and finisher diets [23 to 42 d]). The calculated chemical composition per kilogram of the basal diets was as follows: starter (apparent metabolizable energy corrected for nitrogen [AMEn] 12.6 MJ, crude protein 215 g, lysine 13.2 g, calcium 9 g, and available phosphorus 4.5 g), grower (AMEn 12.9 MJ, crude protein 195 g, lysine 11.9 g, calcium 8.4 g, and available phosphorus 4.2 g) and finisher (AMEn 13.3 MJ, crude protein 180 g, lysine 10.5 g, calcium 7.6 g, and available phosphorus 3.8 g). All diets included coccidiostat (Maxiban G160, Elanco Animal Health, Elli Lilly and Company, Clinton, Indiana, USA). The PP used in this study (Anco FIT - Poultry, Anco Animal Nutrition Competence, GmbH, Sankt Poelten, Austria) was a proprietary mixture of phytogenic substances marketed as a “gut agility activator” with an active ingredient concentration of 70 g/kg. The PP used in this study (Anco FIT-Poultry, Anco Animal Nutrition) consisted of functional flavouring substances of ginger, lemon balm, oregano and thyme on functional carriers 1m558 bentonite and clinoptilolite.

2.2. Tissue sample collection for subsequent analyses

The sample collection procedure and all subsequent analyses were carried out according to Mountzouris et al. (2019) and are briefly presented below. At the end of the experiment (d 42), 10 birds per treatment (i.e. 2 birds per floor pen) were randomly selected and euthanized neatly according to EU 63/2010. Broilers were dissected and the duodenum, jejunum, ileum and ceca were carefully separated and immediately snap-frozen in liquid nitrogen followed by storage in −80 °C, until further analysis.

2.3. RNA isolation and reverse transcription to cDNA

The middle section (15 cm) of duodenum, jejunum, ileum and one of the ceca from each bird sampled were longitudinally opened and the luminal digesta was removed. Afterwards, the sections were thoroughly washed twice in 30-mL ice cold phosphate buffered saline (PBS)-ethylene diamine tetraacetic acid (EDTA; 10 mmol/L; pH = 7.2) solution and each mucosal layer was scraped off with a micro slide and placed in a 1.5-mL sterile Eppendorf tube. Subsequently, total RNA from the duodenal, jejunal, ileal and cecal mucosa was extracted by using NucleoZOL Reagent (Macherey–Nagel GmbH & Co. KG, Düren, Germany), according to the manufacturer's protocol. RNA quantity and quality were determined by spectrophotometry (NanoDrop-1000, Thermo Fisher Scientific, Waltham, United Kingdom). Prior to cDNA synthesis, DNAse treatment was applied. Ten micrograms of RNA were resuspended with 1 U of DNase I (M0303, New England Biolabs

Inc., Ipswich, UK) and 10 μL of 10 × DNAse buffer to a final volume of 100 μL with the addition of DEPC water for 20 min at 37 °C. The DNAse was inactivated by the addition of 1 μL of 0.5 mol/L EDTA at 75 °C for 10 min. RNA integrity was checked by agarose gel electrophoresis.

For cDNA preparation, 500 ng of total RNA from each sample were reverse transcribed to cDNA by PrimeScript RT Reagent Kit (Perfect Real Time, Takara Bio Inc., Shiga-Ken, Japan) according to the manufacturer's recommendations. All cDNA were then stored at −20 °C.

2.4. Quantitative real-time PCR for gene expression in broiler intestinal samples

The following *Gallus gallus* genes were investigated: catalase (*CAT*), superoxide dismutase 1 (*SOD1*), glutathione peroxidase 2 (*GPX2*), heme oxygenase 1 (*HMOX1*), NAD(P)H quinone dehydrogenase 1 (*NQO1*), *Nrf2*, kelch like ECH associated protein 1 (*Keap1*), *NF-κB1*, Toll-like receptor 2 family member B (*TLR2B*), Toll-like receptor 4 (*TLR4*), heat shock 70 kDa protein 2 (*HSP70*), heat shock protein 90 alpha family class A member 1 (*HSP90*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), actin beta (*ACTB*) and peptidylprolyl isomerase A (*PPIA*).

Suitable primers were designed using the Basic Local Alignment Search Tool "Primer3 and BLAST" (Ye et al., 2012) of NCBI and the GenBank sequences deposited therein (National Center for Biotechnology Information U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda MD, 20894 USA) (Table 1). Primers were checked using the PRIMER BLAST algorithm for *Gallus gallus* mRNA databases NCBI transcript reference sequences [refseq_rna] to ensure that there was a unique amplicon. The PCR amplification efficiency of each primer pair was calculated from a respective standard curve using a serial dilution series of pooled cDNA samples, according to Pfaffl (2001).

Real-time PCR was performed in 96-well microplates with a SaCycler-96 Real-Time PCR System (Sacace Biotechnologies s.r.l., Italy) and FastGene IC Green 2 × qPCR universal mix (Nippon Genetics, Japan). Each reaction contained 12.5 ng RNA equivalents as well as 200 nmol/L of forward and reverse primers for each gene. The reactions were initially heated at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 59.5 to 62 °C (depended on the target gene, according to Table 1) for 20 s and 72 °C for 33 s. This was followed by a melt curve analysis to determine the reaction specificity. Each sample was measured in duplicates. Relative expression ratios of target genes were calculated according to Pfaffl (2001) adapted for the multi reference gene normalization procedure using the geometric mean of the linear relative quantities RQ values of the 3 reference genes *GAPDH*, *ACTB* and *PPIA* used according to Hellemans et al. (2007).

2.5. TAC of the intestine

Total antioxidant capacity of intestinal segments was determined by using the ORAC assay (Cao and Prior, 1999) and the results were expressed as concentration of Trolox equivalents TE (mmol/g of mucosa tissue). Briefly, for the ORAC assay, intestinal mucosa samples were thoroughly homogenized 10-fold (i.e., 10%, wt/vol) with PBS (0.1 mol/L; pH = 7) using a tissue grind tube. Subsequently, the homogenate was centrifuged for 10 min at 12,000 × *g* at 4 °C, the supernatant was collected and centrifuged again for 30 min at 50,000 × *g* at 4 °C. Finally, the supernatant was separated

Table 1
Oligonucleotide primers used for the study of gene expression of selected targets by quantitative real-time PCR.

Target	Primer sequence (5′–3′)	Annealing temperature, °C	PCR product size, bp	GenBank accession No. (NCBI Reference Sequence)
GAPDH	F: ACTTTGGCATTGTGGAGGGT R: GGACGCTGGGATGATGTTCT	59.5	131	NM_204305.1
ACTB	F: CACAGATCATGTTTGAGACCTT R: CATCACAATACCAGTGGTACG	60	101	NM_205518.1
PPIA	F: CCTGCTTCCACCGGATCAT R: CCGTTGTGGCGCGTAAA	60	64	NM_001166326.1
CAT	F: ACCAAGTACTGCAAGGCGAA R: TGAGGGTTCCTCTTCTGGCT	60	245	NM_001031215
SOD1	F: AGGGGGTCATCCACTTCC R: CCCATTTGTGTTGTCTCCAA	60	122	NM_205064.1
GPX2	F: GAGCCCAACTTCACCCTGTT R: CTTCAGGTAGGCGAAGACGG	62	75	NM_001277854.1
HMOX1	F: ACACCCGCTATTTGGGAGAC R: GAACTTGGTGGCGTTGGAGA	62	134	NM_205344.1
NQO1	F: GAGCGAAGTTCAGCCCAGT R: ATGGCGTGGTTGAAAGAGGT	60.5	150	NM_001277619.1
Nrf2	F: AGACGCTTTCTTCAGGGGTAG R: AAAAACTTCACGCCTTGCCC	60	285	NM_205117.1
Keap1	F: GGTTACGATGGGACGGATCA R: CACGTAGATCTTGCCCTGGT	62	135	XM_025145847.1
NF-κB1	F: GAAGGAATCGTACCGGGAACA R: CTCAGAGGGCCTTGTGACAGTAA	59	131	NM_205134.1
TLR2B	F: CTTGGAGATCAGAGTTTGGA R: ATTTGGGAATTTGAGTGCTG	62	238	NM_001161650.1
TLR4	F: GTCTCTCCTTCCTTACCTGCTGTTC R: AGGAGGAGAAAGACAGGGTAGGTG	64.5	187	NM_001030693.1
HSP70	F: ATGCTAATGGTATCCTGAACG R: TCCTCTGCTTTGTATTTCTCTG	60	145	NM_001006685.1
HSP90	F: CACGATCGCACTCTGACCAT R: CTGTCACCTTCTCCGCAACA	60	196	NM_001109785.1

F = forward; R = reverse; *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase; *ACTB* = actin, beta; *PPIA* = peptidylprolyl isomerase A; *CAT* = catalase; *SOD1* = superoxide dismutase 1; *GPX2* = glutathione peroxidase 2; *HMOX1* = heme oxygenase 1; *NQO1* = NAD(P)H quinone dehydrogenase 1; *Nrf2* = nuclear factor (erythroid-derived 2)-like 2; *Keap1* = kelch like ECH associated protein 1; *NF-κB1* = nuclear factor kappa B subunit 1; *TLR2B* = Toll-like receptor 2 family member B; *TLR4* = Toll-like receptor 4; *HSP70* = heat shock 70 kDa protein 2; *HSP90* = heat shock protein 90 alpha family class A member 1.

and stored at −80 °C until analysis following appropriate dilution with PBS.

2.6. Statistical analysis

Experimental data on intestinal gene expression and TAC were based on individual broilers. All data were initially checked for normality and subsequently analyzed with the general linear model (GLM) − ANOVA procedure using the SPSS for Windows statistical package program, version 8.0.0 (SPSS Inc., Chicago, IL). Statistically significant effects were further analyzed and means were compared using Tukey's honest significant difference (HSD) multiple comparison procedure. Statistical significance was determined at $P \leq 0.05$. Linear (lin) and quadratic (quad) response patterns to dietary PP inclusion level were studied using polynomial contrasts.

3. Results

3.1. Profile of selected gene expression along the broiler intestine

The relative expression results of the *Keap1/Nrf2/ARE* pathway genes *CAT*, *SOD1*, *GPX2*, *HMOX1*, *NQO1*, *Nrf2*, *Keap1* along the intestine are presented per intestinal segment in Table 2. In addition, the relative expression results of *NF-κB1*, *TLR2B*, *TLR4*, *HSP70*, *HSP90* are shown per intestinal segment in Table 3.

3.1.1. Duodenal mucosa

In the duodenum, significant changes between the experimental treatments were shown for the expression of *SOD1* ($P = 0.027$), *GPX2* ($P = 0.032$), *HMOX1* ($P = 0.046$), *NQO1* ($P = 0.050$) and *Keap1* ($P = 0.027$), as shown in Table 2. Polynomial contrast analysis showed that the relative expression of the genes above displayed quadratic patterns of increase with increasing PP inclusion level. In particular, expression of *SOD1* ($P_{quad} = 0.003$), *GPX2* ($P_{quad} = 0.004$), *HMOX1* ($P_{quad} = 0.037$), *NQO1* ($P_{quad} = 0.017$) and *Keap1* ($P_{quad} = 0.004$) in treatments PP-750 and PP-1000 generally reached the highest levels compared primarily to CON and secondarily to PP-2000. In addition, the expression of *NF-κB1* in duodenum differed ($P = 0.007$) between the experimental treatments, displaying a linear ($P_{lin} = 0.011$) pattern of decrease with increasing PP level (Table 3).

3.1.2. Jejunal mucosa

In the jejunum, *SOD1* ($P = 0.026$), *GPX2* ($P = 0.013$) and *NQO1* ($P = 0.011$) expression differed between experimental treatments (Table 2). More specifically, *SOD1* and *NQO1* expression in treatment PP-750 were significantly higher compared to CON but did not differ from treatments PP-1000 and PP-2000. Expression of

Table 2
Relative expression of the *Keap1/NRf2/ARE* pathway genes along the intestinal mucosa of 42-d-old broilers.

Item[1]	Treatments[2]				SEM	*P*-values[3]		
	CON	PP-750	PP-1000	PP-2000		P_{ANOVA}	P_{lin}	P_{quad}
Duodenum								
CAT	1.02	1.44	0.94	0.94	0.200	0.219	0.243	0.143
SOD1	0.83^{a}	1.29^{bc}	1.35^{c}	0.92^{ab}	0.200	0.027	0.614	0.003
GPX2	1.14^{ab}	1.28^{ab}	1.98^{b}	0.84^{a}	0.292	0.032	0.830	0.004
HMOX1	0.78^{a}	1.20^{ab}	1.44^{b}	1.17^{ab}	0.224	0.046	0.055	0.037
NQO1	0.86^{a}	1.24^{b}	1.20^{b}	1.07^{ab}	0.146	0.050	0.193	0.017
Nrf2	1.02	1.20	1.20	1.04	0.231	0.782	0.925	0.307
Keap1	0.83^{a}	1.39^{b}	1.31^{b}	0.99^{ab}	0.202	0.027	0.554	0.004
Jejunum								
CAT	1.02	1.22	1.00	1.08	0.197	0.661	0.961	0.641
SOD1	0.90^{a}	1.40^{b}	0.96^{ab}	1.06^{ab}	0.171	0.026	0.937	0.111
GPX2	0.69^{a}	1.71^{b}	1.89^{b}	0.98^{ab}	0.405	0.013	0.075	0.004
HMOX1	0.90	1.00	1.14	1.27	0.147	0.085	0.011	0.845
NQO1	0.86^{a}	1.23^{b}	1.11^{ab}	1.14^{ab}	0.156	0.011	0.155	0.124
Nrf2	1.37	1.67	1.38	1.24	0.406	0.757	0.592	0.448
Keap1	0.92	1.11	1.13	1.07	0.157	0.535	0.353	0.259
Ileum								
CAT	0.96	1.13	1.11	1.15	0.193	0.738	0.362	0.627
SOD1	0.91	1.09	1.18	1.10	0.182	0.301	0.246	0.309
GPX2	0.89	1.13	1.38	1.27	0.236	0.057	0.068	0.301
HMOX1	0.98	1.14	1.34	1.12	0.203	0.370	0.340	0.184
NQO1	0.87	1.22	1.37	1.32	0.199	0.067	0.022	0.158
Nrf2	0.99	1.46	1.10	1.28	0.258	0.294	0.548	0.427
Keap1	0.87	1.09	1.39	1.12	0.190	0.076	0.086	0.080
Ceca								
CAT	1.03	1.54	1.67	0.90	0.453	0.263	0.858	0.053
SOD1	0.70^{a}	1.40^{b}	1.42^{b}	1.06^{ab}	0.255	0.023	0.181	0.006
GPX2	0.78^{A}	1.11^{AB}	1.13^{AB}	1.59^{B}	0.212	0.006	0.001	0.684
HMOX1	0.89^{a}	1.60^{b}	1.31^{ab}	0.94^{a}	0.245	0.019	0.860	0.003
NQO1	0.74^{a}	1.29^{b}	1.13^{ab}	1.32^{b}	0.198	0.022	0.016	0.210
Nrf2	0.76^{a}	1.27^{ab}	1.69^{b}	1.12^{ab}	0.286	0.024	0.107	0.012
Keap1	0.72^{A}	1.52^{C}	1.26^{BC}	1.00^{AB}	0.162	<0.001	0.254	<0.001

Keap1 = kelch like ECH associated protein 1; *Nrf2* = nuclear factor (erythroid-derived 2)-like 2; *ARE* = antioxidant response element; SEM = pooled standard error of means; *CAT* = catalase; *SOD1* = superoxide dismutase 1; *GPX2* = glutathione peroxidase 2; *HMOX1* = heme oxygenase 1; *NQO1* = NAD(P)H quinone dehydrogenase 1.

[1] Relative expression ratios of target genes were calculated according to Pfaffl (2001) adapted for the multi-reference genes normalization procedure according to Hellemans et al. (2007) using glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)*, actin, beta (*ACTB*) and peptidylprolyl isomerase A (*PPIA*) as reference genes.

[2] CON, basal diet with no phytogenic premix (PP) addition; PP-750, basal diet containing PP at 750 mg/kg diet; PP-1000, basal diet containing PP at 1,000 mg/kg diet; PP-2000, basal diet containing PP at 2,000 mg/kg diet. Data represent treatment means from $n = 10$ broilers analyzed per treatment.

[3] The statistical analysis tests the differences between treatments (ANOVA) and the linear (lin) and quadratic (quad) effect of PP inclusion levels (polynomial contrasts). Means within the same row with different superscripts (a, b) differ at $P < 0.05$ and with different superscripts (A, B, C) differ at $P < 0.01$.

Table 3
Relative gene expression of transcription factor *NF-κB1*, *TLR* and *HSP* along the intestinal mucosa of 42-d-old broilers.

Item[1]	Treatments[2]				SEM	*P*-values[3]		
	CON	PP-750	PP-1000	PP-2000		P_{ANOVA}	P_{lin}	P_{quad}
Duodenum								
NF-κB1	1.07^{AB}	1.28^{B}	0.93^{A}	0.82^{A}	0.129	0.007	0.011	0.087
TLR2B	1.38	0.62	1.43	0.99	0.561	0.542	0.897	0.780
TLR4	0.75	1.65	1.26	1.20	0.353	0.108	0.394	0.062
HSP70	1.03	1.76	0.98	0.95	0.282	0.143	0.264	0.063
HSP90	1.16	1.19	1.11	0.85	0.177	0.241	0.086	0.267
Jejunum								
NF-κB1	1.12	1.12	1.08	0.99	0.202	0.917	0.518	0.781
TLR2B	1.11	0.69	0.73	0.87	0.364	0.656	0.563	0.282
TLR4	0.91	1.31	1.10	1.20	0.264	0.484	0.437	0.430
HSP70	1.09	1.76	1.17	0.87	0.452	0.136	0.174	0.546
HSP90	1.18	1.54	1.01	0.85	0.256	0.134	0.069	0.157
Ileum								
NF-κB1	1.19	1.23	0.96	0.99	0.225	0.514	0.215	0.975
TLR2B	1.83	0.66	1.44	1.76	0.697	0.375	0.572	0.540
TLR4	0.76	1.50	1.29	1.24	0.349	0.207	0.274	0.121
HSP70	1.21	1.77	1.03	0.88	0.396	0.258	0.179	0.207
HSP90	1.24	1.62	1.02	0.93	0.329	0.214	0.150	0.312
Ceca								
NF-κB1	0.95^{AB}	1.33^{B}	1.15^{B}	0.72^{A}	0.140	0.001	0.057	<0.001
TLR2B	1.27	1.94	1.03	0.85	0.355	0.081	0.063	0.098
TLR4	1.16^{AB}	1.64^{B}	0.97^{A}	0.78^{A}	0.248	0.009	0.027	0.061
HSP70	1.80^{b}	1.55^{ab}	0.98^{ab}	0.67^{a}	0.382	0.011	0.002	0.912
HSP90	0.89	1.26	1.07	1.25	0.249	0.425	0.277	0.597

NF-κB1 = nuclear factor kappa B subunit 1; *TLR* = Toll-like receptor; *HSP* = heat shock protein; SEM = pooled standard error of means.
[1] Relative expression ratios of target genes were calculated according to Pfaffl (2001) adapted for the multi-reference genes normalization procedure according to Hellemans et al. (2007) using glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)*, actin, beta (*ACTB*) and peptidylprolyl isomerase A (*PPIA*) as reference genes.
[2] CON, basal diet with no phytogenic premix (PP) addition; PP-750, basal diet containing PP at 750 mg/kg diet; PP-1000, basal diet containing PP at 1,000 mg/kg diet; PP-2000, basal diet containing PP at 2,000 mg/kg diet. Data represent treatment means from $n = 10$ broilers analyzed per treatment.
[3] The statistical analysis tests the differences between treatments (ANOVA) and the linear (lin) and quadratic (quad) effect of PP inclusion levels (polynomial contrasts). Means within the same row with different superscripts (a, b) differ at ($P < 0.05$) and with different superscripts (A, B) differ at $P < 0.01$.

GPX2 showed a quadratic ($P_{quad} = 0.004$) pattern of increase with increasing PP inclusion level with PP-750 and PP-1000 being higher than CON.

3.1.3. Ileal mucosa

In the ileum there were no significant ($P > 0.05$) differences between the experimental treatments for any of the genes studied (Tables 2 and 3).

3.1.4. Cecal mucosa

In the ceca, the expression of *SOD1* ($P = 0.023$), *GPX2* ($P = 0.006$), *HMOX1* ($P = 0.019$), *NQO1* ($P = 0.022$), *Nrf2* ($P = 0.024$) and *Keap1* ($P < 0.001$) differed between the experimental treatments (Table 2). Increasing PP inclusion level resulted in quadratic patterns of increase for *SOD1* ($P_{quad} = 0.006$), *HMOX1* ($P_{quad} = 0.003$), *Nrf2* ($P_{quad} = 0.012$) and *Keap1* ($P_{quad} < 0.001$), and *GPX2* and *NQO1* expression displayed linear patterns of increase ($P_{lin} = 0.001$ and 0.016; respectively). Compared to CON, the relative expression levels were highest for PP-1000 and PP-2000 for the first (i.e. *SOD1* and *Nrf2*) and second (i.e. *GPX2* and *NQO1*) set of genes above, respectively. In the case of *HMOX1* and *Keap1*, treatment PP-750 had the highest expression compared to CON. Finally, significant differences were shown for *NF-κB1* ($P = 0.001$), *TLR4* ($P = 0.009$) and *HSP70* ($P = 0.011$). In all 3 cases (Table 3), increasing PP inclusion level resulted in reduced expression, in a linear fashion for *TLR4* ($P_{lin} = 0.027$) and *HSP70* ($P_{lin} = 0.002$) and a quadratic one for *NF-κB1* ($P_{quad} < 0.001$).

3.2. Profile of intestinal TAC

Significant differences in the TAC between treatments were found in the duodenum ($P = 0.011$) and the ceca ($P = 0.050$) as shown in Table 4. Increasing PP inclusion level resulted in patterns of TAC increase in a linear ($P_{lin} = 0.007$) and quadratic ($P_{quad} = 0.046$) manner in the duodenum and in a quadratic ($P_{quad} = 0.014$) manner in the ceca. In both cases treatment PP-1000 had the highest TAC compared to CON.

4. Discussion

This study aimed to generate new baseline information on the mechanisms of phytogenic function at the broiler gut level under normal-physiological and non-challenge experimental conditions. In particular, PP inclusion level effects on an array of critical genes for host protection against oxidation (*CAT*, *SOD*, *GPX2*, *HMOX1*, *NQO1*, *Nrf2* and *Keap1*), stress (*HSP70* and *HSP90*) and inflammation (*NF-κB1*, *TLR2* and *TLR4*) were profiled along the broiler intestine. Overall, the results revealed consistent and significant changes for the majority of the genes analyzed. In particular, the modulation of gene expression was shown to depend on the PP inclusion level and the intestinal segment concerned.

Firstly, it was shown that PP inclusion increased the expression of most of the *Keap1/Nrf2/ARE* pathway genes studied, primarily in the duodenum and the ceca and secondarily in the jejunum. In particular, the up-regulated genes *SOD1* and *GPX2* as well as *NQO1* and *HMOX1* code for enzymes known for their antioxidant and detoxification functions, respectively. For example, SOD catalyzes the conversion of the extremely reactive superoxide (O^{-}_{2}) into stabilized dioxygen (O_2) and hydrogen peroxide (H_2O_2). In turn, GPX2 can reduce H_2O_2 to water and lipid peroxides to their analogous alcohols (Stefanson and Bakovic, 2014; Lee et al., 2017). Furthermore, HMOX1 is regarded as a highly protective enzyme that directly inhibits pro-inflammatory cytokines and activates anti-inflammatory ones. In addition, HMOX1 catalyzes the

Table 4
Total antioxidant capacity (TAC) along the intestinal mucosa of 42-d-old broilers mmol TE per gram mucosa tissue.

Item	Treatments[1]				SEM	*P*-values[2]		
	CON	PP-750	PP-1000	PP-2000		P_{ANOVA}	P_{lin}	P_{quad}
Duodenum	37.76[a]	47.86[ab]	54.94[b]	50.45[ab]	4.981	0.011	0.007	0.046
Jejunum	52.67	52.99	55.75	48.35	7.781	0.819	0.681	0.488
Ileum	43.19	46.45	44.47	52.23	7.092	0.598	0.270	0.657
Ceca	29.41[a]	39.98[ab]	44.58[b]	35.45[ab]	5.569	0.050	0.193	0.014

TE = Trolox equivalents.
[1] CON, basal diet with no phytogenic premix (PP) addition; PP-750, basal diet containing PP at 750 mg/kg diet; PP-1000, basal diet containing PP at 1,000 mg/kg diet; PP-2000, basal diet containing PP at 2,000 mg/kg diet. Data represent treatment means from $n = 10$ broilers analyzed per treatment.
[2] The statistical analysis tests the differences between treatments (ANOVA) and the linear (lin) and quadratic (quad) effect of PP inclusion levels (polynomial contrasts). Means within the same row with different superscripts (a, b) differ at $P < 0.05$.

degradation of heme into carbon monoxide, free iron and bilirubin. In turn, carbon monoxide acts as NF-κB1 inhibitor, whereas bilirubin acts as an antioxidant (Ahmed et al., 2017; Lee et al., 2017). Finally, NQO1 protects against generation of reactive oxygen intermediates that are formed during oxidative cycling of quinones and it has also been shown to scavenge superoxide directly and to maintain stability of proteins critical for cell function (Lee et al., 2017; Ross and Siegel, 2018).

In this work, in addition to the cytoprotective genes induced, PP inclusion modulated the expression of *Nrf2* and *Keap1*. The Nrf2 is a key transcription factor considered as the master regulator of cellular antioxidant response and xenobiotic metabolism (Bocci and Valacchi, 2015; Huang et al., 2015). The protein Keap1 is the Nrf2 inhibitor, known to play by far the greatest role in regulating the *Nrf2* signaling pathway (Baird and Dinkova-Kostova, 2011). In particular, the Keap1, due to its high reactive thiol content, is a highly sensitive redox sensor that is localized near the plasma membrane where it encounters exogenous electrophiles and the products of lipid peroxidation (Stefanson and Bakovic, 2014). Under physiological conditions most Nrf2 are sequestered in the cytoplasm by Keap1 and targeted for constant proteasomal degradation. When dissociation of the Keap1-Nrf2 complex occurs due to covalent modification or oxidation of critical cysteine residues in Keap1 and/or direct Nrf2 activation, the Nrf2 translocates into the nucleus and binds to *ARE* leading to transcription of cytoprotective genes (Stefanson and Bakovic, 2014; Lee et al., 2017).

It is interesting to note that in the duodenum, jejunum and ceca the up-regulated *Keap1/Nrf2/ARE* pathway genes were largely characterized by a quadratic pattern of increase with increasing PP inclusion level. Linear patterns of increase were additionally shown but were limited to 3 cases (i.e. *HMOX1* in the jejunum; *GPX2* and *NQO1* in the ceca). Generally, it was shown that compared to CON treatment, the highest gene expression levels occurred in treatments PP-750 and PP-1000 and in only 2 cases (i.e. cecal *GPX2* and *NQO1*) in PP-2000. Therefore, under the non-challenge experimental conditions used, PP inclusion levels of 750 and 1,000 mg/kg diet sufficed to increase the relevant cytoprotective gene expression. The finding above highlighted and confirmed the necessity of optimizing PP inclusion levels with respect to the targeted biological responses. This has also been proven in the cases of broiler performance, meat antioxidant capacity, nutrient digestibility, gut microbiota and gut integrity previously reported (Mountzouris et al., 2011, 2019; Paraskeuas et al., 2017; Paraskeuas and Mountzouris, 2019a).

In this study, *NF-κB1* expression in the duodenum and the ceca was reduced in a quadratic fashion with increasing PP inclusion level. The NF-κB1 protein is a key transcription factor that regulates cellular immune responses to infection and higher order oxidative stress by coordination of a pro-inflammatory response (Zhang and Ghosh, 2001; Stefanson and Bakovic, 2014; Wardyn et al., 2015). In this study, when the *Keap1/Nrf2/ARE* pathway genes were up-regulated, *NF-κB1* expression was down-regulated. This finding is in agreement with the general notion that activation (i.e. up-regulation) of the *Keap1/Nrf2/ARE* pathway inhibits *NF-κB1* activation resulting in increased protection against oxidative stress and inflammation (Sahin et al., 2013; Wardyn et al., 2015; Lee at al., 2017; Huang and Lee, 2018). Indeed, several studies reviewed by Allen and Tresini (2000) and Sahin et al. (2013) have shown that antioxidant enzymes (e.g. CAT, SOD and GPX) block activation of *NF-κB1* by various stimuli such as H_2O_2 and inflammatory cytokines and factors (e.g. interleukin 1 and tumor necrosis factor α), most likely by preventing the degradation of the NF-κB1 inhibitor-alpha protein (IkBα). The latter sequesters NF-κB1 in the cytoplasm (Stefanson and Bakovic, 2014; Huang and Lee, 2018) and blocks the translocation of NF-κB1 to the nucleus and the subsequent transcription initiation of inflammatory components. In addition, HMOX1 and Keap1 inhibit NF-κB1 activation, the former via the production of carbon monoxide (Ahmed et al., 2017; Lee et al., 2017) and the latter by blocking the IkBα proteosomal degradation (Stefanson and Bakovic, 2014).

Moreover, it is known that NF-κB1 activation and the subsequent production of an inflammatory response could be limited by down-regulation of Toll-like receptor (TLR) signaling (Shibolet and Podolsky, 2007; Kawai and Akira, 2007; Lillehoj and Lee, 2012; Keestra et al., 2013). In this study, in addition to cecal *NF-κB1*, cecal *TLR4* expression was linearly reduced with increasing PP inclusion level. Generally, the reduction of *TLR* expression levels could be essential for limiting inflammation and a number of phytogenic components have been identified to be involved in reducing *TLR* signaling (Lillehoj and Lee, 2012; Peri and Calabrese, 2014). For example, administration of phytogenics such as thymol and carvacrol have been shown to reduce expression levels of ileal *TLR2* (Du et al., 2016) and *TLR4* in cecal tonsils (Lu et al., 2014). We have recently shown that administration of phytogenics based on menthol and anethole (Paraskeuas and Mountzouris, 2019a) as well as carvacrol, anethol and limonen (Paraskeuas and Mountzouris, 2019b) reduced cecal *TLR2* expression. Expression of *TLR* in the gut depends on multifactor host interactions including microbiota composition and multiple stressor challenges (MacKinnon et al., 2009; Lu et al., 2014 Du et al., 2016). In this sense, the down-regulation of cecal *TLR4* may be the result of PP induced changes in the abundance of Gram-negative lipopolysaccharide (LPS) producing bacteria (e.g. *Escherichia coli* and *Salmonella* spp.) in the ceca that merit further investigation.

In this work, cecal *HSP70* expression was reduced in a linear fashion with increasing PP inclusion level. HSP are a group of evolutionarily conserved proteins that are expressed as a response to physical, chemical, or biological stresses and play an important

role in the protection and repair of cells and tissues (Gu et al., 2012; Sohn et al., 2012; Hao and Gu, 2014). For example, HSP70 has been shown to be capable of protecting the intestinal mucosa from heat-stress injury by improving antioxidant capacity and inhibiting the lipid peroxidation production (Gu et al., 2012). Since, HSP serve as a classical marker of stress in animals (Sahin et al., 2013; Lee et al., 2017), then the reduced cecal *HSP70* expression in this work combined with the rest of the findings discussed earlier, could point to a PP potential to contribute towards an improved intestinal environment.

Furthermore, the intestinal profile analysis showed that the duodenum and the ceca were the most responsive intestinal sites to modulation by PP inclusion, followed by the jejunum. Although not directly comparable, intestinal site-specific events were also shown by an earlier study assessing phytogenic effects on *ARE*-regulated genes at the jejunum and the ceca (Mueller et al., 2012). In order to explain the intestinal site specificity seen in the present study, the specific knowledge about the absorption and metabolism kinetics of the PP active components is currently rather limited. For example, absorption in the proximal gut has been reported in the cases of carvacrol and thymol (Michiels et al., 2008; Brenes and Roura, 2010), whereas absorption in the large intestine was reported the case of quercetin (Aura et al., 2002; Suzuki and Hara, 2011). Furthermore, limited evidence on the fate of the absorbed phytogenics in the proximal gut suggests that phytogenic components get metabolized and/or eliminated by the kidneys without significant deposition in the body (Lee et al., 2004; Michiels et al., 2008). In this study, given the diminishing effect of PP inclusion on the responses assessed from the proximal (i.e. duodenum) to the distal (i.e. ileum) small intestine, it could be postulated that the PP bioactive components get absorbed proximally and this explains why the duodenum is more responsive compared to the jejunum and ileum. In addition, it could be possible that PP metabolites get re-introduced in the duodenum via the enterohepatic circulation. Furthermore, according to the known reflux of uric substances in birds (Sacranie et al., 2012), urinary excreted phytogenic compounds and their metabolites may get refluxed back to the ceca. The latter could explain the strong responsiveness of the ceca in PP inclusion, in a similar manner to the phytogenic impact reported for broiler cecal micro-ecology (Paraskeuas and Mountzouris, 2019 a, b).

Finally, it was shown that the most responsive gut segments to PP inclusion were additionally characterized by higher TAC, denoting an overall improved resistance to oxidation. Generally, there is good evidence that the PP constituents may enhance antioxidant capacity when administered in broiler diets. Relevant to literature examples include studies with ginger (Zhang et al., 2009; Zidan et al., 2016), lemon balm (Marcincakova et al., 2011), thyme or thymol and/or oregano or carvacrol alone (Botsoglou et al., 2002; Luna et al., 2010; Hashemipour et al., 2013) or in combination with other phytogenics (Paraskeuas et al., 2016). Furthermore, it has been recently shown that the PP inclusion used in this work improved liver, breast meat and thigh meat TAC (Mountzouris et al., 2019).

The importance of priming the *Keap1/Nrf2/ARE* pathway prior to oxidative stress or xenobiotic encounter has been postulated to increase cellular fitness to respond more robustly to oxidative assaults without activating more intense *NF-κB1* mediated inflammatory responses (Stefanson and Bakovic, 2014). Considering all the above together, PP inclusion has resulted in a consistent expression increase of cytoprotective genes and reduction of stress and inflammation related ones. In addition, changes in the gene expression direction were concomitant with the improved intestinal TAC shown, as well as the enhanced liver, breast meat and thigh meat TAC previously reported (Mountzouris et al., 2019).

5. Conclusion

This work has provided a new insight on PP function in broiler intestine. It was shown that PP inclusion primed the expression of critical genes for host protection against oxidation and down-regulated genes relevant for inflammation (*NF-κB1* and *TLR4*) and stress (*HSP70*). Gut profiling revealed the duodenum and the ceca as the most responsive intestinal segments to modulation by PP, followed by the jejunum. The improved broiler gut response to PP inclusion was additionally documented by the elevated duodenal and cecal TAC at PP inclusion of 1,000 mg/kg diet. Considering all the above together, PP inclusion has resulted in a consistent up-regulation of cytoprotective genes and down-regulation of stress and inflammation related ones. Collectively the study results highlighted phytogenic potential to beneficially stimulate baseline bird cytoprotective response that merits further investigation under various stressor challenges.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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