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Substituting ethoxyquin with tea polyphenols and propyl gallate enhanced feed oxidative stability, broiler hepatic antioxidant capacity and gut health

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ABSTRACT The safety of ethoxyquin has garnered increasing attention. This study evaluated the effects of partially substituting ethoxyquin with tea polyphenols and propyl gallate on feed oxidative stability, hepatic antioxidant properties, intestinal morphology and barrier functions, as well as the antioxidant and anti-inflammatory profiles of the intestinal mucosa in broilers. A total of 351 one-day-old male Arbor Acres Plus broilers were randomly assigned to 3 groups, each comprising 9 replicates with 13 birds per replicate. The treatments included a control group (CON) fed a basal diet, an ethoxyquin group (**EQ**) that received the basal diet supplemented with 120 g/t of ethoxyquin, and a substitution group (**TP**) receiving the basal diet supplemented with 6 g/t of tea polyphenols, 6 g/t of propyl gallate, and 30 g/t of ethoxyquin. In vitro experiments showed that both EQ and TP supplementation significantly reduced the acid value (**AV**), peroxide value (**POV**), and total oxidation value (**TOV**) of the feeds, with the TP group exhibiting lower AV and TOV than the EQ group. In vivo assessments revealed no significant differences in growth performance among the groups. Additionally, the TP group exhibited significantly higher glutathione peroxidase activity, increased glutathione content, and elevated protein expression of Keap1, Nrf2, and NQO1 in the liver compared to the control group ($P < 0.05$). Moreover, dietary TP significantly increased liver catalase activity, glutathione content, and NQO1 protein levels compared to the EQ group ($P < 0.05$). Both additives effectively reduced malondialdehyde levels in the intestinal mucosa by approximately 50% ($P < 0.05$) through the activation of the *Nrf2/ARE* pathway, as indicated by increased mRNA expression of *TXN, CAT, GPX1*, and *GPX4* ($P < 0.05$). Furthermore, compared to the control group, the TP group exhibited greater villus height and villus height-to-crypt depth ratio (VCR) in the jejunum, as well as elevated VCR in the ileum ($P < 0.05$). The TP group also achieved the lowest serum levels of diamine oxidase activity, D-lactate and lipopolysaccharide contents among all groups ($P < 0.05$). The inclusion of both EQ and TP increased the mRNA expression of *Occludin, Claudin-1, Mucin-2*, and *E-cadherin* in the jejunum ($P < 0.05$). Moreover, the combination of tea polyphenols and propyl gallate effectively mitigated the proinflammatory effect of ethoxyquin, as evidenced by reductions in *TNF-α, IL-18*, and *IFN-γ* expression, potentially mediated by inhibition of the *TLR-4/MyD88/NF-κB* signaling pathway. In conclusion, this study demonstrates that partially replacing ethoxyquin with tea polyphenols and propyl gallate enhances feed oxidative stability, liver antioxidant capacity, and gut health in broilers, suggesting an efficient alternative with a lower dosage requirement.

Key words: tea polyphenols, propyl gallate, ethoxyquin, antioxidant capacity, gut health

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INTRODUCTION

Lipid-rich components are frequently added to animal feeds to meet the substantial energy requirements of rapidly growing animals. However, these lipid-rich ingredients are prone to oxidation from manufacturing to utilization, which can compromise the nutritional value of the feed, animal health, and product safety. Consumption of an oxidized diet may negatively impact physiological health, leading to diminished production efficiency (Kwek et al., 2022; Zhang et al., 2023). Thus, incorporating antioxidant-enriched substances into the feed is crucial for enhancing oxidative stability and improving the antioxidant status of animals.

Synthetic antioxidants, such as ethoxyquin, butylated hydroxyanisole (**BHA**), butylated hydroxytoluene (**BHT**), and tertiary butyl hydroquinone, are widely used in animal feed due to their effectiveness, affordability, and stability (Saraswati et al., 2021). Ethoxyquin

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serves as a general antioxidant in animal feed, preventing the oxidation of lipids and fat-soluble vitamins while also offering antimold and preservation benefits (Błaszczyk et al., 2013). However, research indicates that exceeding a certain threshold of ethoxyquin in animal feed may lead to pro-oxidation and negatively impact animal growth and health (Wang et al., 2010; Li et al., 2019). As a result, natural antioxidants are gaining popularity due to health concerns. Safer alternatives to ethoxyquin can reduce reliance on it and enhance the safety of animal products.

Tea polyphenols, derived from natural tea leaves, are widely recognized as an excellent source of antioxidants (Zhang and Tsao, 2016; Truong and Jeong, 2022). This antioxidant regulates various antioxidant enzymes, such as glutathione peroxidase, and activates the Nrf2/ARE defensive pathway (Yan et al., 2020). Another low-toxicity and eco-friendly antioxidant is propyl gallate (**PG**) (Wang et al., 2019; Gulcin, 2020), which exhibits stronger antioxidant properties compared to BHT and BHA (Lin et al., 2009). However, due to its higher cost and the potential for discoloration at doses exceeding 100 mg/kg, PG is often used in combination with other antioxidants (Xu et al., 2021). Nevertheless, few studies have investigated its effects on animals.

Recent studies have highlighted that complex antioxidants typically provide greater benefits compared to individual antioxidants (Wen et al., 2019; Purkait et al., 2020; Haddadi et al., 2023). Exploring safer antioxidant complexes such as tea polyphenols and propyl gallate as substitutes for ethoxyquin may maximize the advantages of each antioxidant, thereby reducing reliance on ethoxyquin and enhancing food safety. This study aimed to decrease ethoxyquin usage by partially substituting it with a combination of tea polyphenols and propyl gallate, investigating the effects on feed oxidation stability, liver antioxidant profile, and gut health in broilers.

MATERIALS AND METHODS

Animals and Experimental Design

A total of 351 one-day-old male AA Plus broilers were randomly assigned to 3 groups, each comprising 9 replicates with 13 chickens per replicate. The control group was fed a basal diet (CON group), while the EQ group received the basal diet supplemented with 200 g/t of ethoxyquin at 60% purity (effective content of 120 g/t). The TP group was provided with the basal diet supplemented with 200 g/t of a compound antioxidant, which included 15% ethoxyquin (effective content of 30 g/t), 3% tea polyphenols (effective content of 6 g/t), and 3% propyl gallate (effective content of 6 g/t). The nutritional analysis of the basal diet is detailed in Table 1. Animal care and experimental procedures followed the guidelines of the Animal Welfare Committee of China Agricultural University (Permit number: AW52704202-1-1) and management practices adhered to the guidelines for Arbor Acres broilers. All broilers were housed in cages with ad libitum access to feed and water using trough-type feeders and nipple drinker lines. Environmental conditions, including temperature and lighting conformed to the AA Plus Broiler Management Guide recommendations.

Feed Oxidation Stability Testing

After a designated storage period, oil samples were extracted to evaluate the oxidation stability index. The specific procedures were as follows: From each of the 3 treatments, 1.2 kg of feed (formulated for 1−21-day-old broilers) was collected and divided into 3 equal portions. These portions were subsequently stored in different locations within the chicken house for duration of 6 wk. During the third and sixth wk, 200 g of feed was sampled, ground into a powder, and passed through a 40-mesh screen before being stored at −20°C. Oil was then extracted to assess oxidation stability indicators. For oil extraction, 100 g of the sample was placed into a wide-mouth bottle, to which 400 mL of petroleum ether was added. The mixture was stirred with a glass rod, and the bottle was tightly sealed. The bottle was then shaken on a reciprocating oscillator for 2 h. The solution and sample were transferred to a 50 mL centrifuge tube and centrifuged at 8,000 r/min for 5 min. The supernatant was collected and transferred to a rotary evaporator where the water bath temperature was maintained below 40°C. The solvent was evaporated under reduced pressure, leaving liquid grease as the final sample. The p-anisidine value (**PAV**) was determined according to Padial-Domínguez et al. (2020). The acid value (**AV**), peroxide value (**POV**), and total oxidation value (**TOV** = **4POV** + **PAV**) were measured following the method described by Ma et al. (2021). Malondialdehyde (**MDA**) content was quantified using commercial kits (cat# BC0025, Solarbio).

Growth Performance

Birds were weighed on d 1, 21, and 42 and feed intake was recorded from day 1 to d 21 and from d 22 to d 42. The average body weight gain (**BWG**) per cage was calculated by subtracting the initial average weight from the final average weight. The feed conversion ratio (**FCR**) was calculated by dividing the total feed intake by the total body weight gain of each pen, including weights of any dead or culled birds. The average feed intake (**AFI**) was corrected by subtracting the feed intake of dead or culled birds (calculated as the product of their body weight gain and FCR) from the total feed intake of each cage, and then divided by the number of remaining birds.

Sample Collection

A healthy chick of average weight was selected from each replicate, stunned with electric shock, and bled via the carotid artery. The small intestine was then

Table 1. Dietary ingredients and nutrient composition of basal diet.

Dietary ingredients (%)	1−21 d	22−42 d	Nutrient composition[3]	1−21 d	22−42 d
Corn	52.35	64.22	Metabolizable energy, Kcal/kg	2,950	3,150
Soybean meal	38.71	24.15	Crude protein, %	22.0	19.0
Corn gluten meal	1.31	5.00	Lys, %	1.31	1.08
Soybean oil	3.06	2.80	Met, %	0.48	0.48
Dicalcium phosphate	1.79	1.12	Met + Cys, %	0.96	0.86
Limestone	1.30	0.88	Thr, %	0.86	0.72
Sodium chloride	0.35	0.20	Val, %	0.89	0.84
Sodium bicarbonate	0.00	0.25	Calcium, %	1.02	0.65
Choline chloride (50%)	0.20	0.15	Nonphytate phosphorus, %	0.39	0.28
L-Lysine hydrochloride (98.5%)	0.26	0.33			
DL-Methionine (98%)	0.31	0.24			
L-Threonine (98.5%)	0.05	0.11			
L-Valine (98.5)	0.00	0.03			
L-Isoleucine (90%)	0.03	0.09			
L-Arginine (98%)	0.02	0.19			
Phytase (10,000U/g)	0.03	0.02			
Mineral premix[1]	0.20	0.20			
Vitamin premix[2]	0.03	0.02			
Total	100.00	100.00			

[1]The mineral premix provided the following per kg of diets: Cu, 16 mg; Zn, 110 mg; Fe, 80 mg; Mn, 120 mg; Se, 0.30 mg; I, 1.50 mg.
[2]The vitamin premix provided the following per kg of diets: vitamin A, 15,000 IU, vitamin D3, 3,600 IU; vitamin E, 30 IU; vitamin K3, 3.00 mg; vitamin B2, 9.60 mg; vitamin B12, 0.03 mg; biotin, 0.15 mg; folic acid, 1.50 mg; pantothenic acid, 13.80 mg; nicotinic acid, 45 mg.
[3]Nutrient and amino acid composition of cereals and protein sources were analyzed using near-infrared spectroscopy prior to feed formulation. The nutrient composition of the basal diet is based on calculated values.

removed, and approximately 1 cm sections from the middle of the duodenum, jejunum, and ileum were collected and fixed in 4% paraformaldehyde solution for morphological analysis. Liver tissue, jejunal tissue, and jejunal mucosa were collected and stored at −80°C for further analysis.

Intestinal Morphology

Tissue sections fixed in 4% paraformaldehyde were embedded in paraffin, sectioned, dehydrated, and stained with hematoxylin and eosin by Sevier Biotechnology Co., Ltd. (Wuhan, China). Villus height (**VH**), crypt depth (**CD**), and the villus height-to-crypt depth ratio (**VCR**) were measured using a Leica DM 750 optical microscope (Leica Microsystems, Wetzlar, Germany).

Antioxidant Capacity of Liver and Intestinal Mucosa

The activities of total antioxidant capacity (**T-AOC**), superoxide dismutase (**SOD**), catalase (**CAT**), and glutathione peroxidase (**GSH-Px**), glutathione (**GSH**) and MDA in the liver were assessed and normalized to total protein content using kits from Nanjing Jiancheng Bioengineering Institute (catalog numbers: A015-2, A001-3, A007-1-1, A003-1, A005-1, A006-2, A045-4). Additionally, the levels of T-AOC, CAT, SOD, and MDA in the intestinal mucosa were measured with kits from Beijing Solarbio Science & Technology Co., Ltd. (catalog numbers: BC1315, BC0205, BC5165, BC0025) and were also normalized to total protein content using the BCA kit from Shanghai Biyuntian Biotechnology Co., Ltd. (catalog number: P0010).

Gene Expression

Total RNA was extracted using RNAiso Plus (catalog number 9108, Takara, Japan) and reverse transcribed into cDNA using the Prime Script^{TM} RT Reagent Kit with gDNA Eraser (catalog number RR047A, Takara, Japan). Real-time quantitative fluorescence reaction was conducted with the SYBR Premix Ex Taq kit (catalog number RR420A, Takara, Japan) on an ABI7500 quantitative PCR instrument (Applied Biosystems). All primer sequences for target genes are presented in Table 2. Relative RNA abundance was assessed as previously described (Yang et al., 2023). The relative expression levels of each gene were normalized to β-actin and analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

Liver tissue (100 mg) was homogenized in 1 mL of RIPA lysis solution (Beijing Solarbio Biotech, China) with 1 mM phenylmethylsulfonyl fluoride (**PMSF**) using a SCIENTZ-12 high-throughput tissue homogenizer (Xinzhi Biotech, China). Cell disruption was further facilitated with a tissue disruptor (Xinzhi Biotech, China). Following incubation on ice for 30 min, the samples were centrifuged at 12,000 × g for 15 min to collect the supernatant. Soluble proteins were separated by SDS-PAGE gel electrophoresis (100 V for 2 h), transferred to a nitrocellulose (NC) membrane (120 V for 40 min), and blocked with 5% skimmed milk powder (Sangon Biotech, China). The NC membrane was then probed with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 1 h at room temperature. Western blot bands were scanned and analyzed using a fully automated chemiluminescence imaging system, with band density normalized to β-actin content. The primary antibodies used included Keap1 (G2): sc-365626, Nrf2(A10): sc-363949, HO-1(A3): sc-

Table 2. Sequences of primers used for the quantitative real-time PCR analysis.

Gene	Primer sequences (5'-3')*	Accession number
β-actin	F: TTGTTGACAATGGCTCCGGT R: TCTGGGCTTCATCACCAACG	NM_205518.1
ZO-1	F: CTTCAGGTGTTTCTCTTCCTCCTC R: CTGTGGTTTCATGGCTGGATC	XM_413773
ZO-2	F: GGCAGCTATCAGACCACTCT R: GCTGGGAAGGAAGAGAACCT	NM_204918.1
ZO-3	F: CGGCAAGATCGCCAACATTA R: CCATGAGGGTCGTAGTCCTC	XM_015299757.2
Claudin-1	F: TACAGCCCTTGGCCAATACA R: CCAAGAAACAACCACCAGCA	NM_001013611.2
Occludin	F: CGCAGATGTCCAGCGGTTACT R: CAGAGCAGGATGACGATGAGGAA	NM_205128.1
Mucin-2	F: AATGCTGAGTTCTTGCCTAA R: TGTTGCAGTTCATATCCTGGT	XM_001234581.3
E-cadherin	F: CCTCCAGGATGTGAATGACAACG R: ATGCTCCAGTGCTGCCTTGAAG	NM_001039258.2
Keap1	F: CATCGGCATCGCCAACTT R: TGAAGAACTCCTCCTGCTTGGA	XM_025145847.1
Nrf2	F: GAGAAAGCCTTGCTGGCTCA R: TGAAGTATCTGTGCTCTGCGAA	NM_205117.1
HO-1	F: CTTCGCACAAGGAGTGTTAAC R: CATCCTGCTTGTCCTCTCAC	NM_205344.1
TXN	F: GGCAATCTGGCTGATTTTGA R: ACCATGTGGCAGAGAAATCA	NM_205453.1
SOD1	F: GGCAATGTGACTGCAAAGGG R: ATGCAGTGTGGTCCGGTAAG	NM_205064.1
SOD2	F: TACAGCTCAGGTGTCGCTTC R: GCGAAGGAACCAAAGTCACG	NM_204211.1
CAT	F: GCGCCCCGAACTATTATCCA R: ATACGTGCGCCATAGTCAGG	NM_001030762.3
GPX1	F: TGCGCCCGATGTTTTCAAAG R: AACGTTACCCAGACTCACGG	NM_001277853.2
GPX4	F: GGGTGAAGTTCGACATGTTCAG R: GTTCCACTTGATGGCATTCCC	NM_001346448.1
IFN-γ	F: CTCGCAACCTTCACCTCACCATC R: CAGGAACCAGGCACGAGCTTG	NM_205149.1
IL-6	F: GAACGTCGAGTCTCTGTGCTAC R: CACCATCTGCCGGATCGT	NM_204628
IL-1β	F: CAGCCTCAGCGAAGAGACCTT R: ACTGTGGTGTGCTCAGAATCC	NM_204524
TNF-α	F: CCCCTACCCTGTCCCACAA R: TGAGTACTGCGGAGGGTTCAT	NM_204267
IL-18	F: GTGTGTGCAGTACGGCTTAG R: TCCACTGCCAGATTTCACCT	NM_204608.1
TLR-4	F: CCACTATTCGGTTGGTGGAC R: ACAGCTTCTCAGCAGGCAAT	NM_001030693.1
MyD88	F: TGCAAGACCATGAAGAACGA R: TCACGGCAGCAAGAGAGATT	NM_001030962.3
NF-κB	F: TGGAGAAGGCTATGCAGCTT R: CATCCTGGACAGCAGTGAGA	NM_205134.1

*F, forward primer; R, reverse primer.Note: Zonula occluden-1 (*ZO-1*), zonula occluden-2 (*ZO-2*), zonula occluden-3 (*ZO-3*), Claudin-1, Occludin, mucin 2 (*Mucin-2*), epithelial cadherin (*E-cadherin*), nuclear factor erythroid 2-related factor 2 (*Nrf2*), heme oxygenase 1 (*HO-1*), thioredoxin (*TXN*), superoxide dismutase 1 (*SOD1*), superoxide dismutase 2 (*SOD2*), catalase (*CAT*), glutathione peroxidase 1 (*GPX1*), glutathione peroxidase 4 (*GPX4*), interferon γ (*IFN-γ*), interleukin 6 (*IL-6*), interleukin 1β (*IL-1β*), tumor necrosis factor α (*TNF-α*), interleukin 18 (*IL-18*), toll-like receptor 4 (*TLR-4*), myeloid differentiation factor88 (*MyD88*), nuclear factor of κ-light chain of enhancer-activated B cells (*NF-κB*).

136960 and NQO1(A5): sc-271116 (Santa Cruz Biotechnology, Inc). The antibeta actin secondary antibody (#7076) was from Cell Signaling Technology.

Statistical Analysis

One-way ANOVA was performed using SPSS 26.0 software. Multiple comparisons were conducted using the Duncan test. Percentages were compared with an inverse chord correction. The results are expressed as mean and pooled standard errors of the means (SEM). Differences were considered significant at $P < 0.05$, and trends were indicated at $0.05 \leq P < 0.1$. Graphs were generated using GraphPad Prism 9.

RESULTS

Effects of EQ and TP on the Oxidative Stability of Feeds (In Vitro)

Dietary supplementation with EQ and TP significantly reduced the AV ($P = 0.012$), POV ($P = 0.003$), and TOV ($P = 0.005$) of the feed after 3 wk of storage (Table 3). Additionally, the PAV and TOV of the TP group were significantly lower than those of the EQ group ($P < 0.05$). After 6 wk, compared to the control group, the TP-supplemented diet exhibited significantly decreased AV ($P = 0.002$), POV ($P = 0.031$), and TOV ($P = 0.088$). Moreover, the TP group showed lower AV than the EQ group ($P < 0.05$).

Effects of EQ and TP on the Growth Performance of Broilers

While the addition of TP resulted in slight improvements in BWG and FCR of broilers during both 1 to 21-day and 22 to 42-day periods, the differences between the groups were not statistically significant ($P > 0.05$, Table 4).

Effects of EQ and TP on Liver Antioxidant Capacity of Broilers

The addition of TP significantly increased GSH-Px activity ($P = 0.01$) and GSH content ($P = 0.019$) in the liver, while showing a trend towards increasing SOD activity ($P = 0.069$) (Table 5). Furthermore, CAT activity and GSH content in the TP group were significantly higher than in the EQ group ($P < 0.05$). TP supplementation also elevated the expression levels of Keap1, Nrf2, and NQO1 proteins in the liver ($P < 0.05$) (Protein electrophoresis bands are shown in Supplementary Figure S1). Additionally, NQO1 protein expression in the EQ group was significantly lower than in both the control and TP groups ($P < 0.001$).

Effects of TP and EQ on Intestinal Antioxidant Capacity in Broilers

Dietary supplementation with EQ or TP significantly reduced MDA content ($P < 0.001$). The EQ supplementation tended to increase T-AOC in the jejunal mucosa ($P < 0.1$) (Table 6). Moreover, the expression of antioxidant-related genes was assessed to investigate the underlying mechanisms (Table 6). Dietary inclusion of both EQ and TP increased the expression of *TXN, CAT, GPX1*, and *GPX4* genes ($P < 0.05$). Furthermore, the

Table 3. Effects of different antioxidants on the oxidative stability of feeds.

Item	Stored 3 wk					Stored 6 wk				
	CON	EQ	TP	SEM	*P*-value	CON	EQ	TP	SEM	*P*-value
AV, mmol/kg	15.49[a]	13.69[b]	12.46[b]	0.503	0.012	16.94[a]	15.21[b]	12.98[c]	0.615	0.002
POV, meq/kg	1.57[a]	0.52[b]	0.50[b]	0.192	0.003	1.64[a]	0.89[b]	0.63[b]	0.184	0.031
MDA, μmol/mL	19.06	18.85	17.32	0.420	0.190	17.51	16.68	16.06	0.558	0.630
PAV	3.65[b]	5.16[a]	2.63[b]	0.414	0.009	5.38	3.69	3.41	0.584	0.378
TOV	9.94[a]	7.23[b]	4.62[c]	0.842	0.005	11.95[a]	7.23[ab]	5.94[b]	1.227	0.088

Notes: N = 3. AV, acid value; POV, peroxide value; MDA, malondialdehyde; PAV, p-anisidine value; TOV, total oxidative value.
[ab]Different superscript letters indicate significant differences ($P < 0.05$). CON, basal diet; EQ, basal diet + 120 g/t ethoxyquin; TP, basal diet + 30 g/t ethoxyquin, 6 g/t tea polyphenols, and 6 g/t propyl gallate.

Table 4. Effects of different antioxidants on the growth performance of broilers.

Item	BWG (g)	AFI (g)	FCR
1-21d			
CON	803	1,023	1.27
EQ	788	995	1.26
TP	805	1,011	1.26
SEM	3.8	4.9	0.005
P-value	0.153	0.069	0.431
22−42d			
CON	1,661	2,904	1.75
EQ	1,703	2,928	1.72
TP	1,696	2,945	1.74
SEM	17.8	22.7	0.009
P-value	0.609	0.774	0.387
1−42d			
CON	2,459	3,920	1.59
EQ	2,492	3,923	1.57
TP	2,502	3,960	1.58
SEM	18.4	24.9	0.005
P-value	0.634	0.783	0.169

Notes: N = 9. BWG, body weight gain; AFI, average feed intake; FCR, feed conversion ratio.
[ab]Different superscript letters indicate significant differences ($P < 0.05$). CON, basal diet; EQ, basal diet + 120 g/t ethoxyquin; TP, basal diet + 30 g/t ethoxyquin, 6 g/t tea polyphenols, and 6 g/t propyl gallate.

Nrf2 expression in the EQ group was significantly higher than in the other groups ($P = 0.003$), while *Keap1* expression in the TP group was significantly lower compared to the other groups ($P = 0.028$).

Table 5. Effects of different antioxidants on liver antioxidant capacity of broilers.

Item	CON	EQ	TP	SEM	*P*
TAOC (μmol/mL)	72.15	79.33	75.06	1.686	0.224
SOD (U/mgprot)	29.26	29.49	31.02	0.342	0.069
CAT (U/mgprot)	9.08[a]	7.99[b]	9.53[a]	0.191	<0.001
GSH-Px (U/mgprot)	31.46[b]	34.37[a]	35.84[a]	0.652	0.010
GSH (μmol/gprot)	11.89[b]	11.44[b]	14.25[a]	0.458	0.019
MDA (nmol/mgprot)	0.48	0.42	0.51	0.022	0.239
Keap1	1.00[b]	1.08[b]	1.20[a]	0.026	0.003
Nrf2	1.00[b]	1.26[a]	1.25[a]	0.039	0.004
NQO1	1.00[b]	0.79[c]	1.12[a]	0.035	<0.001
HO-1	1.00	1.10	1.08	0.025	0.255

Notes: N = 8. Total antioxidant capacity (T-AOC), the activity of superoxide dismutase (SOD) and catalase (CAT) and glutathione peroxidase (GSH-Px), and the levels of glutathione (GSH) and malondialdehyde (MDA) in the liver. Relative expression of antioxidant-related proteins (Keap1, Nrf2, HO-1, and NQO1) in the liver (Protein electrophoresis bands are shown in Supplementary Figure S1).
[ab]Different superscript letters indicate significant differences ($P < 0.05$). CON, basal diet; EQ, basal diet + 120 g/t ethoxyquin; TP, basal diet + 30 g/t ethoxyquin, 6 g/t tea polyphenols, and 6 g/t propyl gallate.

Effects of TP and EQ on Intestinal Morphology of Broilers

Supplementation with EQ and TP resulted in a significant increase in jejunal VH and CD ($P < 0.001$) (Table 7). Additionally, TP supplementation tended to increase the VCR in the jejunum ($P = 0.062$). Ileal VH in the EQ group ($P = 0.048$) and VCR in the TP group ($P = 0.014$) were significantly higher than in the control group. Additionally, the TP group displayed lower CD than the EQ group ($P = 0.028$). Notably, broilers in the TP group exhibited the highest VCR in the duodenum ($P > 0.05$), jejunum ($P < 0.1$), and ileum ($P < 0.05$).

Effects of TP and EQ on Intestinal Barrier and Inflammation-related Cytokines of Broilers

Dietary supplementation with TP significantly decreased serum DAO activity ($P < 0.001$), D-Lactate, and LPS levels in broiler chickens ($P < 0.05$), while EQ supplementation only significantly reduced serum LPS levels ($P < 0.05$) (Table 8). Gene expression related to the intestinal barrier was further analyzed to investigate the mechanisms. The results indicated that

Table 6. Effects of different antioxidants on intestinal mucosal antioxidant status of broilers.

Item	CON	EQ	TP	SEM	*P*-value
MDA (nmol/mgprot)	0.44[a]	0.19[b]	0.22[b]	0.027	<0.001
T-AOC (μmol/gprot)	100.70	112.20	109.08	2.274	0.090
CAT (U/mgprot)	42.74	44.20	41.13	2.307	0.874
SOD (U/mgprot)	12.96	13.15	14.83	0.621	0.422
Keap1	1.00[a]	0.95[a]	0.68[b]	0.057	0.028
NRF2	1.00[b]	2.20[a]	1.31[b]	0.174	0.003
Ho-1	1.00	1.18	0.85	0.069	0.115
TXN	1.00[b]	1.93[a]	2.01[a]	0.163	0.004
SOD1	1.00	1.09	0.78	0.078	0.245
SOD2	1.00	1.38	1.12	0.079	0.153
CAT	1.00[b]	1.92[a]	1.79[a]	0.157	0.022
GPX1	1.00[c]	2.51[a]	1.40[b]	0.169	<0.001
GPX4	1.00[b]	1.98[a]	2.04[a]	0.187	0.005

Notes: N = 8. Malondialdehyde (MDA) content, total antioxidant capacity (T-AOC) and the activity of superoxide dismutase (SOD) and catalase (CAT) in the jejunal mucosa. Relative mRNA expression of antioxidant-related genes (*Keap1, Nrf2, HO-1, TXN, SOD1, SOD2, CAT, GPX1* and *GPX4*) in the jejunal mucosa.
[ab]Different superscript letters indicate significant differences ($P < 0.05$). CON, basal diet; EQ, basal diet + 120 g/t ethoxyquin; TP, basal diet + 30 g/t ethoxyquin, 6 g/t tea polyphenols, and 6 g/t propyl gallate.

Table 7. Effects of different antioxidants on the intestinal morphology of broilers.

	Duodenum			Jejunum			Ileum		
Item	VH	CD	VCR	VH	CD	VCR	VH	CD	VCR
CON	1,277	197	6.36	841[b]	139[b]	5.70	548[b]	110[ab]	5.02[b]
EQ	1,341	206	6.01	1,254[a]	183[a]	6.36	627[a]	122[a]	5.42[ab]
TP	1,399	214	6.69	1,161[a]	178[a]	6.86	589[ab]	105[b]	5.75[a]
SEM	39.3	5.7	0.240	50.4	5.2	0.205	13.4	2.8	0.106
P-value	0.466	0.500	0.527	<0.001	<0.001	0.062	0.048	0.028	0.014

Notes: N = 9. VH: villus height, CD: crypt depth, VCR: villus height-to-crypt depth ratio.
[ab]Different superscript letters indicate significant differences ($P < 0.05$). CON, basal diet; EQ, basal diet + 120 g/t ethoxyquin; TP, basal diet + 30 g/t ethoxyquin, 6 g/t tea polyphenols, and 6 g/t propyl gallate.

Table 8. Effects of different antioxidants on the intestinal barrier and inflammation-related cytokines of broilers.

Item	CON	EQ	TP	SEM	*P*-value
DAO (ng/mL)	1.55[a]	1.43[a]	0.81[b]	0.081	<0.001
D-Lactate (μmol/mL)	1.82[a]	1.61[ab]	1.47[b]	0.057	0.037
LPS (EU/L)	113.0[a]	94.4[b]	86.2[b]	3.551	0.014
ZO-1	1.00[a]	1.02[a]	0.84[b]	0.032	0.049
ZO-2	1.00[b]	1.52[a]	1.43[ab]	0.097	0.042
ZO-3	1.00	0.95	0.95	0.068	0.815
Occludin	1.00[c]	1.92[a]	1.75[b]	0.117	<0.001
Claudin-1	1.00[c]	3.12[a]	1.38[b]	0.247	<0.001
Mucin-2	1.00[b]	3.71[a]	2.39[a]	0.375	<0.001
E-cadherin	1.00[b]	1.93[a]	1.91[a]	0.139	0.002
IFN-γ	1.00	1.97	1.31	0.163	0.058
IL-6	1.00	1.68	0.67	0.194	0.459
IL-1β	1.00	0.97	0.61	0.115	0.159
TNF-α	1.00[a]	1.13[a]	0.64[b]	0.075	0.007
IL-18	1.00[c]	2.43[a]	1.43[b]	0.174	<0.001
TLR-4	1.00[a]	1.00[a]	0.53[b]	0.085	0.008
MyD88	1.00	1.44	0.99	0.093	0.066
NF-KB	1.00[b]	2.20[a]	1.06[b]	0.149	<0.001

Notes: N = 8. Serum diamine oxidase (DAO) activity, D-Lactate and lipopolysaccharide (LPS) levels. Relative mRNA expression of intestinal barrier related genes (*ZO-1, ZO-2, ZO-3, Occludin, Claudin-1, Mucin-2* and *E-cadherin*) in the jejunum. Relative mRNA expression of inflammation-related genes (*IFN-γ, IL-6, IL-1β, TNF-α* and *IL-18, TLR-4, MyD88*, and *NF-κB*) in the jejunal mucosa.
[ab]Different superscript letters indicate significant differences ($P < 0.05$). CON, basal diet; EQ, basal diet + 120 g/t ethoxyquin; TP, basal diet + 30 g/t ethoxyquin, 6 g/t tea polyphenols, and 6 g/t propyl gallate.

supplementing the diet with TP and EQ significantly increased the relative mRNA expression of *Occludin, Claudin-1, Mucin-2,* and *E-cadherin* in the jejunum ($P < 0.05$). However, *ZO-1* gene expression in broilers in the TP group was lower compared to other groups ($P < 0.05$).

Dietary addition of EQ significantly increased the expression of *IL-18* ($P < 0.001$), while there was a tendency to decrease the expression of *IFN-γ* ($P = 0.058$), compared with the control group (Table 8). In contrast, TP supplementation reduced the expression of *TNF-α* ($P = 0.007$), *IL-18* ($P < 0.001$), and *IFN-γ* ($P = 0.058$) compared to the EQ group. Although the expression of *IL-6* and *IL-1β* was not statistically significant, their expression was numerically lower in the TP group compared to the EQ group ($P > 0.05$). The expression of the *TLR-4/MyD88/NF-κB* pathway showed that the TP group exhibited lower expression of *TLR-4* ($P = 0.008$), *MyD88* ($P = 0.066$), and *NF-κB* ($P < 0.001$) compared to the EQ group.

DISCUSSION

High-fat feed is highly susceptible to lipid oxidation and rancidity. To prevent oxidation and maintain feed quality, ethoxyquin is widely used in poultry feed (Błaszczyk et al., 2013). However, concerns about the safety and appropriate dosage of ethoxyquin are growing (Błaszczyk and Skolimowski, 2015; Zhang et al., 2021). As a result, nutritionists are increasingly exploring natural antioxidants as potential alternatives for synthetic ones (Yan et al., 2020). Plant-derived compounds, such as tea polyphenols and propyl gallate, demonstrate strong antioxidant properties, indicating that their application could potentially replace ethoxyquin and reduce dependency on it.

Free fatty acids and peroxides, indicated by AV and POV, respectively, are primary oxidation products in the early stage of lipid oxidation (Sharma et al., 2014). Additionally, TOV provides a comprehensive assessment of both primary and secondary oxidation products (Padial-Domínguez et al., 2020). This study demonstrated that supplementing diets with EQ and TP reduced AV, POV, and TOV levels, indicating their ability to inhibit lipid oxidation at the initial stages. Moreover, TP proved more effective than EQ. While oxidation of dietary fats can lead to oxidative stress in animals, which negatively impacts production performance, the inclusion of antioxidants helps to mitigate these effects (Zhou et al., 2021). However, this study found that neither EQ nor TP significantly improved growth performance of broilers, despite slight improvement in BWG and FCR. This may be due to the dosage used, which might have been insufficient to produce a notable impact on growth performance (Xu et al., 2024b; Elokil et al., 2024).

The liver exhibits strong antioxidant capacity primarily through enzyme systems, such as SOD, GSH-Px, and CAT (Zha et al., 2020). Additionally, GSH not only directly eliminates free radicals and excess oxides but also serves as a co-factor for GSH-Px in the metabolism of hydrogen peroxide and lipid peroxides (Ghosh et al., 2018). Incorporating tea polyphenols into the diet has been linked to increased GSH-Px and T-AOC levels, along with a significant reduction in MDA in birds (Chen et al., 2024). Similarly, this study found that TP supplementation increased SOD activity, GSH-Px activity, and GSH content. Conversely, EQ supplementation notably reduced CAT activity, consistent with prior

findings that high levels of ethoxyquin lower liver CAT activity (Elokil et al., 2024). The Nrf2/ARE pathway regulates the expression of antioxidant proteins, with Nrf2 being a key transcription factor in animal tissues, primarily existing in an inactive state in the cytoplasm, bound by Keap1 and targeted for proteasomal degradation (Halliwell, 2024). However, under oxidative stress, Nrf2 becomes phosphorylated, dissociates from Keap1, enters the nucleus, and activates the antioxidant response element (ARE), triggering the transcription of genes like SOD, CAT, GPX, NQO1, HO-1, and TXN (Chatterjee et al., 2020). By increasing Nrf2 levels, endogenous antioxidant defenses can be strengthened. Tea polyphenols are known to boost antioxidant capacity in animals by activating the Nrf2/ARE pathway (Wu et al., 2017; Wang et al., 2020). This study confirms that dietary TP supplementation enhances liver antioxidant capacity in broilers via this pathway, as indicated by increased SOD, CAT, and GSH-Px activity, and higher levels of GSH, Nrf2, and NQO1.

Broiler intestines are particularly vulnerable to oxidative stress, particularly in intensive rearing systems (Ducatelle et al., 2018). Therefore, preserving the antioxidant capacity of the intestinal mucosa is crucial for protection. MDA, as the final product of lipid peroxidation, serves as an indicator of oxidative damage and reflects the homeostasis of the redox system (Jiang et al., 2016). This study found that dietary supplementation with EQ and TP reduced MDA levels in the jejunal mucosa by 64.6% and 49.9%, respectively, compared to the control. Prior studies suggest tea polyphenols boosts Nrf2-related gene expression and reduces intestinal oxidative stress (Song et al., 2019; Zhang et al., 2020). Our research found that adding natural antioxidants activated the *Nrf2/ARE* pathway, increasing the expression of *CAT, GPX1, GPX4*, and *TXN*. CAT plays a key role in neutralizing excess hydrogen peroxide to prevent cellular damage, while GPXs use GSH to convert hydroperoxides into water and alcohol (Ng et al., 2007). GPx4 plays a crucial role in reducing membrane lipid peroxides, and a deficiency or absence of GPX4 can lead to ferroptosis, a form of cell death characterized by membrane lipid peroxidation (Yang et al., 2014). Additionally, TXN is essential for scavenging reactive oxygen species, repairing DNA damage, and inhibiting apoptosis (Liu et al., 2022). The enhanced antioxidant capacity in the intestinal mucosa, along with the protective effects of TXN and GPX4 against cell death and damage could be crucial for developing intestinal villi, as supported by the results of intestinal morphology. Interestingly, EQ and TP activate the Nrf2/ARE pathway through different mechanisms: EQ directly increases Nrf2 expression, while TP reduces Keap1 expression, raising free Nrf2 levels. Although previous research by Ma et al. (2024) showed that tea polyphenols downregulates Keap1 expression, further investigation is needed to fully understand the regulatory mechanisms involved.

Improved VH and VCR are indicators of enhanced intestinal absorption and secretion capacity (Xie et al., 2021). Previous studies have shown that tea polyphenols elevated intestinal VH and VCR while decreasing CD (Wei et al., 2021). In this study, TP supplementation resulted in increased VH and VCR in broilers. Notably, the TP group exhibited a higher VCR across all intestinal segments, suggesting that TP promotes the development of intestinal villi, which may contribute to improved digestion and absorption of nutrients. The intestinal epithelium functions as a protective barrier, shielding the host from harmful stimuli and pathogens (Benjamin et al., 2013). Intestinal barrier permeability can be evaluated by measuring serum D-lactate, DAO, and LPS levels, which rise with increased permeability (Li et al., 2022). This study found that TP supplementation reduced serum D-Lactate, LPS levels, and DAO activity, suggesting decreased intestinal permeability in broilers. Tight junction proteins, including Claudins, ZOs, and Occludin, along with adhesion molecules such as E-cadherin, are essential for maintaining the integrity of epithelial barrier (Turner, 2009). This study found that TP and EQ enhanced the expression of *ZO-2, Occludin, Claudin-1, Claudin-2*, and *E-cadherin*. The intestinal mucus layer, primarily composed of Mucin-2, acts as the primary physical barrier between bacteria and host epithelial cells, protecting against intestinal bacteria and toxins (Birchenough et al., 2016; Wlodarska et al., 2017). Our study showed that dietary supplementation with both EQ and TP significantly increased *Mucin-2* expression. Interestingly, the TP group exhibited better intestinal barrier integrity compared to the EQ group, despite the absence of a significant increase in barrier-related gene expression, suggesting that other factors may have contributed to the enhanced intestinal integrity.

High levels of inflammatory cytokines, such as IFN-γ and TNF-α, can lead to increased shedding and apoptosis of intestinal cells, compromising epithelial integrity (Kiesslich et al., 2007; Mazzarella et al., 2008). In this study, the EQ group showed elevated expression of proinflammatory cytokines (*IFN-γ* and *IL-18*), consistent with previous studies (Elokil et al., 2024). Conversely, intestinal *TNF-α* and *IL-18* levels in broilers in the TP group were significantly lower than those in the EQ group, likely due to the inflammation-reducing properties of tea polyphenols (Liu et al., 2017; Xu et al., 2024b). The reduced expression of inflammatory factors in the TP group may contribute to improved intestinal permeability in broilers. When Toll-like receptor 4 (TLR-4) on immune cells detects LPS from Gram-negative bacteria, the MyD88 protein activates transcription factors like NF-κB through a protein kinase cascade, leading to the release of cytokines like TNF-α, IL-1β, and IL-6 (Wu et al., 2017). This study found that TP significantly inhibited the activation of the TLR-4/MyD88/NF-κB pathway in the intestinal mucosa compared to EQ, aligning with earlier research showing that tea polyphenols mitigate inflammation via this pathway (Xu et al., 2024a).

The results indicated that partially replacing ethoxyquin with tea polyphenols and propyl gallate led to improved feed oxidation stability and enhanced broiler

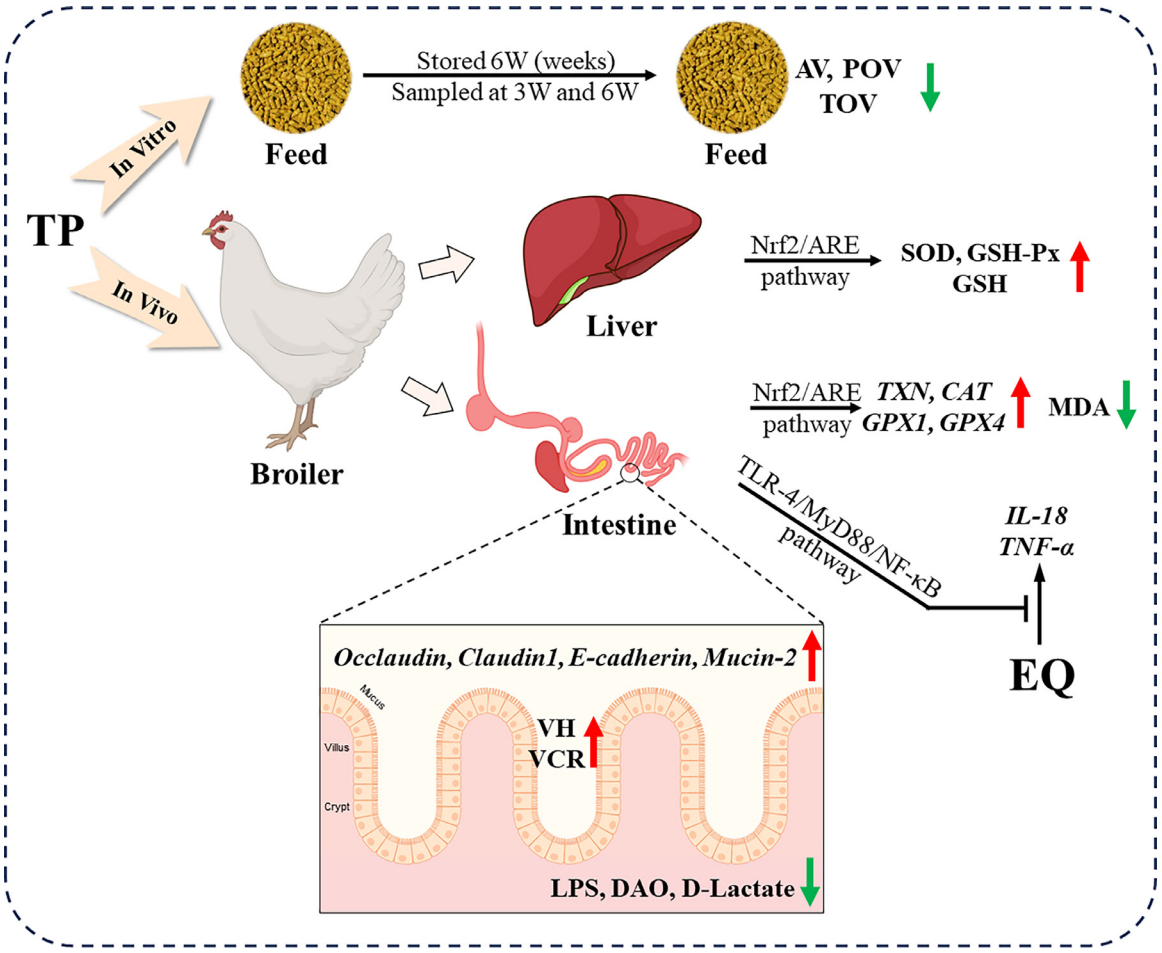

Figure 1. Effects of substituting part of ethoxyquin with tea polyphenols and propyl gallate in broiler diets on feed oxidation stability and broiler health. In vitro results indicated that this substitution led to improved oxidative stability of the feed. *In vivo* experimental results demonstrated that TP improved the antioxidant capacity of broiler liver and intestines via the Nrf2/ARE pathway, while also enhancing intestinal morphology and barrier function. Furthermore, the combination of tea polyphenols with propyl gallate mitigated the proinflammatory effects of ethoxyquin through the TLR-4/MyD88/NF-κB pathway. Green arrows represent decreases and red arrows represent increases. Abbreviations: AV, acid value; POV, peroxide value; TOV, total oxidative value; VH, villus height; VCR, villus height-to-crypt depth ratio. CON, basal diet; EQ, basal diet + 120 g/t ethoxyquin; TP, basal diet + 30 g/t ethoxyquin, 6 g/t tea polyphenols, and 6 g/t propyl gallate. The graphical abstract was created using BioRender (www.biorender.com).

health. Specifically, TP was found to enhance the antioxidant profile of broiler liver and intestine by activating the Nrf2/ARE pathway (Figure 1). Moreover, the combination of tea polyphenols and propyl gallate effectively mitigated the proinflammatory effects of ethoxyquin by targeting the TLR-4/MyD88/NF-κB pathway. However, further optimization of the formulation, including adjustments to dose combinations and ratios, is needed to maximize the overall effect.

CONCLUSION

The study revealed that replacing a portion of ethoxyquin (90 g/t) with tea polyphenols and propyl gallate (6 g/t each) resulted in superior outcomes compared to using ethoxyquin alone at a dosage of 120 g/t. This substitution not only mitigated feed oxidation in vitro but also enhanced liver antioxidant capacity, improved intestinal barrier function, and reduced inflammation in vivo. Overall, the findings suggest that a combination of 6 g/t tea polyphenols, 6 g/t propyl gallate, and 30 g/t ethoxyquin serves as an effective alternative to the addition of 120 g/t ethoxyquin alone.

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Ethics Statement: The animal study was reviewed and approved by the Animal Ethics Committee of the China Agricultural University (Permit Number: AW52704202-1-1).

DISCLOSURES

No conflict of interest exists in the submission of this manuscript, and the manuscript is approved by all authors for publication. I would like to declare that the work described was original research that has not been

previously published and is not under consideration for publication elsewhere, in whole or in part.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.104368.

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