Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola
ISSN 1516-635X Apr - Jun 2018 / v.20 / n.2 / 333-342
http://dx.doi.org/10.1590/1806-9061-2017-0610

Effect of Oxidized Soybean Oils on Oxidative Status and Intestinal Barrier Function in Broiler Chickens

■Author(s)

Tan L[I,II]
Rong D[II]
Yang Y[I]
Zhang B[I]

[I] State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing, 100193, China.
[II] Guizhou Animal Husbandry and Veterinary Research Institute, Guizhou Academy of Agricultural Sciences, Guiyang, Guizhou, 550005, China.

■Mail Address

Corresponding author e-mail address
Bingkun Zhang
China Agricultural University,
Beijing, 100193, China.
Tel: +86-10-62734978
Email: bingkunzhang@126.com

■Keywords

Intestinal barrier function, inflammation-related factor, oxidative stress, heat-oxidized oil, broilers.

Submitted: 15/October/2017
Approved: 21/December/2017

ABSTRACT

The objective of this study was to evaluate the effect of oxidized soybean oils on the growth performance, metabolic oxidative status and intestinal barrier function of broiler chickens. A total of 240 one-day-old female broiler chickens were assigned to four dietary treatments with six replicates (cages) of 10 birds each. The dietary treatments comprised of a basal diet supplemented with 4% of: non-oxidized (fresh) soybean oil (control treatment, SNX); lowly-oxidized soybean oil (SLX) (oil heated for 10h at 200°C); moderately-oxidized soybean oil (SMX) (oil heated for 18h at 200°C); or highly-oxidized soybean oil (SHX) (oil heated for 30h at 200°C). Diets and water were offered *ad libitum*. The experiment was lasted 21d.The growth performance of broilers, determined from 1 to 14 d and from 1 to 21 d of age, was not affected by the dietary treatments ($p>0.05$). Broilers fed oxidized soybean oils presented higher corticosterone serum levels compared with those fed non-oxidized oil ($p<0.05$). Higher malondialdehyde (MDA) levels onday14 and 21 ($p<0.05$), and lower total antioxidant capacity (T-AOC) and totalsuperoxide dismutase (T-SOD) values on day 21were obtained in the liver of broiler fed oxidized oils relative to those fed the non-oxidized oil ($p<0.05$). Broilers fed the highly-oxidized soybean oil had higher ($p<0.05$) MDA levels in the jejunum on day 21 compared with those fed non-oxidized soybean oil. Chickens fed moderately- and highly-oxidized soybean oil presented lower ($p<0.05$) T-SOD activity inileal mucosa compared with those fed non-oxidized soybean oil. Ileal mRNA expression of claudin-1 tended to be down regulated by the dietary addition of oxidized oils ($p=0.056$). The mRNA expression of interleukin-22 (IL-22) of broilers fed moderately-oxidized and highly-oxidized soybean oil was higher ($p<0.05$), and the mRNA expression of occludin and catalase was lower ($p<0.05$) than those fed non-oxidized soybean oil. However, the morphology of the jejunal and ileal mucosa was not influenced ($p>0.05$) by the dietary oxidized oil treatments. It was concluded that oxidized oils may cause oxidative stress by reducing intestinal and liver antioxidant capacity; increase intestinal permeability by reducing mRNA expression levels of tight-junction proteins claudin-1 and occludin; and cause inflammation by increasing mRNA expression level of the inflammation-related factor IL-22.

INTRODUCTION

Oils are added into poultry diets to supply energy and essential fatty acids, as a vitamin vehicle, and to alleviated acute heat stress (Mujahid *et al.*, 2009). Vegetable oils contain large amounts of polyunsaturated fatty acid, such as soybean oil, which is susceptible to peroxidation (Liu

et al., 2014). The fatty-acid composition of lipids used in animal feeds is variable. Lipids used in animal feeds may contain various concentrations of primary and secondary lipid peroxidation products, depending on their fatty-acid composition, storage length, storage conditions, and processing (Totani *et al*., 2007; Totani *et al*., 2008). Heated oils contain various amounts of peroxidation products (Zhang *et al.*, 2012), such as 4-hydroxynonenal, hydroperoxide, malondialdehyde, and 2,4-heptadienal (Choe & Min, 2007), which influence oil odor, palatability, and quality (Paul & Mittal, 1997; Poulli *et al.*, 2009; Smyk, 2015).

The consumption of oxidized phosphatidylcholine can cause damage to organs and increase thiobarbituric acid reactive substances levels in the visceral organs of rats (Al-Orf, 2011). The end-product of *n-3* PUFA oxidation, 4-hydroxy-2-hexenal (4-HHE), induced oxidative stress and inflammation in mice and human intestinal Caco-2/TC7 cells (Awada *et al.*, 2012). In multiple animal species (Hayam *et al.*, 1997; Kumagai *et al.*, 2004; Yue *et al.*, 2011; Ehr *et al.*, 2015), oxidized oils decrease feed intake, depress growth, and even cause disease. Feeding oxidized fish oil impaired the growth performance and induced oxidative stress in *Litopenaeus vannamei* (Yang *et al.*, 2015). Feeding auto-oxidized capelin oil impaired growth rates, antioxidant activities, and increased the occurrence of deformed fish in Siberian sturgeon (*Acipenserbaeri*) larvae (Fontagné *et al.*, 2006). Feeding oxidized fats impaired growth performance by increasing gastrointestinal epithelium cell turnover and hepatic cell proliferation, and increasing the concentration of immunoglobulins in intestinal tissue of broilers and pigs (Dibner *et al*., 1996). Feeding heat-oxidized lipids impaired the metabolic oxidative status of young pigs by depleting serum α-T and increasing serum TBARS (Liu*et al*., 2014).

The biological mechanisms to explain these observations are largely unknown and little information has been reported regarding the effect of feeding oxidized soybean oil on the intestinal barrier function of broiler chickens. The following study was conducted to evaluate the effect of oxidized soybean oil on the performance, metabolic oxidative status, and intestinal barrier function of broiler chickens.

MATERIALS AND METHODS

Fish Oil Preparation

Fresh soybean oil was purchased from the supermarket and stored in a freezer at -30°C until use. In order to oxidize soybean oil, vesicles containing the required amount of fresh soybean oil were heated to 200°C for either 10h, 18h, or 30h to produce low-oxidized soybean oil (SLX), moderately-oxidized soybean oil (SMX) or highly-oxidized soybean oil (SHX), respectively. The processed oils were stored at -30°C prior to their addition to feed. No antioxidant was added before or during the manufacturing of the experimental diets.

The oils were analyzed for their peroxide value (PV) and *p*-anisidine value (*p*-AV), according to ISO methods ISO 3960:2001 IDT (ISO, 2001) and ISO 6885:2006 IDT (ISO, 2006), respectively. Malondialdehyde (MDA) concentrationwas analyzed according to the method of Sidwell *et al*. (1953). Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and vitamin E(VE) content of oils were detected by HPLC methods (Cabuk & Kokturk, 2013). Analytical standards of BHA, BHT, TBHQ, and VE were purchased from Sigma-Aldrich (Sigma-Aldrich, USA).

Birds, Housing and Diets

All experimental procedures were reviewed and approved by the Animal Care and Use Committee of China Agricultural University, Beijing, China.

A total of 240 one-day female broiler chicks were obtained from a commercial hatchery, and assigned to four treatments with six replicate cages of 10 birds each, according to a completely randomized design. The treatments consisted of a basal diet based on corn-soybean meal which formulated to meet or exceed the recommended nutritional requirements of broilers (NRC, 1994; Table 1). No antibiotic growth promoters or antioxidants were added to the basal diet. The experimental diets were produced by adding 4% of four different soybean oil products. The soybean oil products included: fresh soybean oil (control treatment, SNX), lowly-oxidized soybean oil (SLX), moderately-oxidized soybean oil (SMX), and highly-oxidized soybean oil (SHX). The trial lasted 21 days during which birds had *ad libitum* access to feed and water. Initial environmental temperature (day 1) was 31°C, and daily reduced by 0.5°C until 21°C was reached. Continuous lighting was provided during the entire experimental period.

Growth Performance

Birds and feed offered were weighed per pen on the day of hatch, and day14 and day 21. Feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR) were calculated for each period.

Table 1 – Ingredients and calculated nutritional composition of the basal diet.

Ingredient (g/kg)	1 to 21d
Corn	531.80
Soybean meal	385.00
Choline chloride, 50%	2.60
Vitamin premix[1]	0.30
Trace minerals[2]	2.00
Soybean oil	40.00
L-Lysine, 99%	0.80
DL-Methionine, 98%	1.90
Dicalcium phosphate	20.00
Limestone	12.50
Sodium chloride	3.10
Calculated nutrient levels	
Metabolizable energy, kcal/kg	2961
Crude protein[3], g/kg	211.0
Calcium, g/kg	10.1
Available phosphorus, g/kg	4.6
Lysine, g/kg	12.1
Methionine, g/kg	5.0

[1]The vitamin premix provided the following per kilogram of complete diet: vitamin A, 9500 IU; vitamin D_3, 2500IU; vitamin E, 30 IU; vitamin K_3, 2.65 mg; vitamin B_1, 2 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.025 mg; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; nicotinic acid 50 mg.

[2]The trace mineral premix provided the following per kilogram of complete diet: copper, 8 mg ($CuSO_4 \cdot 5H_2O$); zinc, 75mg ($ZnSO_4$); iron, 80 mg ($FeSO_4$); manganese, 100 mg ($MnSO_4 \cdot H_2O$), selenium, 0.15 mg (Na_2SeO_3); iodine, 0.35 mg (KI).

[3]Analyzed.

Sample Collection

On days14 and 21, one bird per cage was randomly selected and sacrificed by venous administration of sodium pentobarbital (30mg/kg of body weight) in order to collect blood, jejunal mucosa, ileal mucosa, and liver samples. After the intestines were removed, the digestawas flushed with 4% saline solution, and the mucous membrane was gently scraped to obtain the samples, which were immediately frozen in liquid nitrogen and stored at -35°C until analysis. On d 14 only, a portion of the ileal mucosa sample was immediately frozen and stored at -80°C for mRNA determination. Additionally, on d 14 and d 21, the right lobe of the liver was extracted and stored at -35°C for evaluation of oxidative stress enzyme activity and MDA concentration. On d 14, blood samples were collected by jugular exsanguination. Serum was separated by centrifugation at 3000×g for 10 min at 4°C, and stored at -35°C until analysis.

Serum analysis

Serum corticosterone (CORT) levels were measured using a radioimmunoassay (RIA) kit (Beijing Sino-uk Institute of Biological Technology, Beijing, China) by an automatic biochemical analyzer (Hitachi High-Technologies, Japan).

Oxidative Stress Enzyme Analysis

Intestinal (jejunal/ileal) mucosa and liver samples (~1 g) were homogenized in 10 mL ice-cold saline solution and centrifuged at 20,000×g for 10 min at 4°C. After appropriate dilution, the supernatant fractions were assayed for total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-PX activities), and total antioxidant capacity (T-AOC) and malondialdehyde (MDA) levels using enzymatic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In order to prevent possible enzyme degradation, the samples were kept on ice throughout detection.

Intestinal morphology analysis

The fixed intestinal samples were dehydrated and embedded in paraffin wax, cut into 3-μm sections, and stained with hematoxylin and eosin. Villus height and crypt depth of 10 randomly selected complete villi per sample were measured at x40 magnification. Villus height was estimated by measuring the vertical distance from the villus tip to villus crypt junction. Crypt depth was measured as the vertical distance from the villus crypt junction to the lower limit of the crypt. The villus height/crypt depth ratio was then calculated from these measurements.

Gene Expression Analysis

Total RNA was extracted from ileal mucosa (~100mg) using trizol reagent, in accordance with the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, California, USA). The purity of the isolated total RNA was determined by measuring its optical density at 260 and 280nm. Samples of the extracted total RNA (2 μg) were reverse transcribed using a reverse transcription kit (Invitrogen Life Technologies, Carlsbad, California, USA). The expression levels of targeted genes were measured according to quantitative real-time PCR assay with a 7300 real-time PCR system (Applied Biosystems, Foster City, CA) using Fast Start Universal SYBR Green Master (Roche) after generation of standard curves for each of five selected gene products: claudin-1, occludin, interleukin-22 (IL-22), catalase (CAT), and β-actin. The primer pairs for the amplification of the appropriate cDNA fragments are listed in Table 2. The PCR program consisted of an initial denaturation step for 10 min at 95°C, an amplification step (40 cycles of 1 min at 95°C), an annealing and extension step for 5 min at 60°C, and a final extension step for 10 min 72°C. All measurements were carried out in triplicate, and values were averaged. The PCR products from each

primer pair were subjected to a melting curve analysis in order to confirm amplification specificity. The expression levels of the target genes were calculated using the comparative threshold cycle method (Livak & Schmittgen, 2001) and data were expressed as values relative to the control group.

Table 2 – Oligonucleotide primers used for quantitative real-time PCR of ileal mucosa tissue samples.

Gene	Primer fragment	Accession number
β-actin	5′-GGATTGGAGGCTCTATCCTGG-3′	NM_205518.1
	5′-GTTTAGAAGCATTTGCGGTGG-3′	
Claudin-1	5′-GATGCGGATGGCTGTCTTTG-3′	NM_001013611.2
	5′-GCTGGGTGGGTAGGATGTTTC-3′	
Occludin	5′-GCCGTAACCCCGAGTTGGAT-3′	NM_205128.1
	5′-TGATTGAGGCGGTCGTTGATG-3′	
IL22	5′-ACCCGTATGCTGAGGATGTGG-3′	NM_001199614.1
	5′-CTTGTTCCCTCCCTTCTTTGG-3′	
CAT	5′-AGCAGGTGCCTTTGGCTATT-3′	NM_001031215.1
	5′-CGAGGGTCACGAACTGTATCA-3′	

Statistical Analysis

Data on growth performance were analyzed per pen basis. All other data were analyzed per individual bird. Data were subjected to Levene's test for homogeneity of variances before further statistical analysis, and expressed as mean values and associated standard errors. Data were analyzed by one-way analysis of variance using the procedures of SPSS Version 18.0 statistical software (SPSS Inc, Chicago, Illinois, USA). Differences among means were identified using Duncan's multiple-range test. Differences were considered significant at $p<0.05$.

RESULTS

Chemical Characteristics of the Experimental Soybean Oils

The concentrations of PV, MDA, *p*-AV, BHA, BHT, TBHQ, VE in the four experimental soybean oils are shown in Table 3. BHA, BHT, and TBHQ were not detected in any of the oils. As expected, with increased duration of heating, the peroxide value of the oil gradually increased. Compared with the SNX oil, the concentration of MDA and *p*-AV in the three oxidized oils increased.

Table 3 – Biochemical analysis of the experimental oils[1].

	Value			
Item	SNX	SLX	SMX	SHX
PV (meq/kg)	3.69	25.37	56.83	73.21
MDA (ug/kg)	5.76	53.30	42.91	32.96
p-AV	2.01	280	289	292
BHA (mg/kg)	ND[2]	ND	ND	ND
BHT (mg/kg)	ND	ND	ND	ND
TBHQ (mg/kg)	ND	ND	ND	ND
VE (mg/kg)	2218	380	139	ND

[1]SNX = non-oxidized soybean oil (fresh soybean oil); SLX = lowly-oxidized soybean oil (heated for 10 h); SMX = moderately-oxidized soybean oil (heated for 18 h); SHX = highly-oxidized soybean oil (heated for 30 h).PV = peroxide value; p-AV = p-anisidine value; MDA = malondialdehyde; BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene;TBHQ = tertiary butylhydroquinone;VE = vitamin E.

[2]ND = not detected

Growth Performance

Different oxidation level of soybean oil did not affect broiler growth performance from 1 to 14 d of age and from 1 to 21 d of age ($p>0.05$, Table 4).

Metabolic Oxidative Status

The serum concentration of corticosterone was affected ($p<0.05$) by dietary oil treatment (Table 5). Chickens fed oxidized soybean oil, regardless of the oxidation levels in soybean oil, presented significant higher corticosterone serum levels compared with those fed the non-oxidized soybean oil diet.

Liver MDA and T-AOC concentrations and GSH-PX and T-SOD activities were shown in the Table 5. The content of MDA in the liver on days 14 and

Table 4 – Growth performance of broilers fed diets with the inclusion of fresh soybean oil (SNX), lowly-, moderately-, or highly-oxidized soybean oil (SLX, SMX and SHX) [1, 2].

	1 to 14 d			1 to 21d		
Treatment	BWG(g)	FI (g)	FCR	BWG (g)	FI (g)	FCR
SNX	253	308	1.22	607	790	1.31
SLX	240	298	1.24	622	812	1.31
SMX	232	293	1.27	599	792	1.32
SHX	253	319	1.26	629	848	1.35
SEM	5.60	5.50	0.01	10.47	12	0.02
p-value	0.487	0.394	0.816	0.752	0.359	0.796

[1]Data are presented as the mean value of 10 birds per replicate cage (n = 6 replicate cages). BWG = body weight gain, FI = feed intake, FCR = feed conversion ratio. SEM = standard error of the mean.

[2] Soybean oils were incorporated into the diet at a level of 4%.

[a,b]Means in the same column with different superscript letters are significantly different ($p< 0.05$).

21, and T-AOC and T-SOD activities on day 21were affected by dietary oil treatment (p<0.05). Chickens fed highly-oxidized soybean oil presented higher (p<0.05) MDA levels compared with those fed the non-oxidized soybean oil diet. Both SMX- and SHX-fed birds presented lower (p<0.05) T-AOC and T-SOD levels in the liver on day 21 compared with the SNX-fed birds. The oxidation levels of soybean oil did not affect T-AOC, T-SOD and GSH-PX levels in liver on day 14 (p>0.05).

Table 5 – Serum corticosterone levels and liver levels of antioxidant-related compounds and enzymes of broilers fed diets with fresh or oxidized soybean oils[1, 2].

Treatment	14d					21d			
	Serum CORT (ng/mL)	MDA (nmol/mg prot)	T-AOC (U/mg prot)	GSH-PX (U/mg prot)	T-SOD (U/mg prot)	MDA (nmol/mg prot)	T-AOC (U/mg prot)	GSH-PX (U/mg prot)	T-SOD (U/mg prot)
SNX	8.46^{b}	0.743^{bc}	1.635	14.93	187	0.753^{b}	1.738^{a}	10.93	186^{a}
SLX	13.88^{a}	0.625^{c}	1.424	16.93	180	0.712^{b}	1.625^{ab}	11.04	168^{ab}
SMX	13.43^{a}	0.796^{ab}	1.594	15.18	173	0.632^{b}	1.345^{c}	11.13	154^{bc}
SHX	12.86^{a}	0.885^{a}	1.606	15.57	185	1.089^{a}	1.388^{bc}	10.48	141^{c}
SEM	0.52	0.029	0.048	0.45	2	0.057	0.053	0.15	5
p-value	0.000	0.006	0.141	0.417	0.134	0.014	0.012	0.443	0.000

[1]Data are presented as the mean value of 10 birds per replicate cage (n = 6 replicate cages). SNX = non-oxidized soybean oil (fresh soybean oil); SLX = lowly-oxidized soybean oil (heated for 10 h); SMX = moderately-oxidized soybean oil (heated for 18 h); SHX = highly-oxidized soybean oil (heated for 30 h). Serum CORT = serum corticosterone; MDA = malonaldehyde; T-AOC = totalantioxidant capacity; GSH-PX = glutathione peroxidase; T-SOD = total superoxide dismutase. mg prot =milligram protein. SEM = standard error of the mean.

[2]Soybean oils were included in the diet at a level of 4%.

[a,b]Means in the same column with different superscript letters are significantly different (p<0.05)

The concentrations of MDA in jejunum on day 21 were affected (p<0.05) by dietary oxidized oil treatment (Table 6). The jejunum of chickens fed highly-oxidized soybean oil had higher (p<0.05) MDA concentrations on day 21 compared with those fed non-oxidized soybean oil diet. Dietary oxidized oil treatment did not influence the metabolic oxidative status of the jejunal mucosa on day 14 (p>0.05).

Table 6 – Levels of antioxidant-related compounds in the jejunal mucosa of broilers fed diets with fresh or oxidized soybean oils[1, 2].

Treatment	14d			21d		
	MDA(nmol/ mg prot)	T-AOC(U /mg prot)	T-SOD(U /mg prot)	MDA(nmol/ mg prot)	T-AOC(U /mg prot)	T-SOD(U /mg prot)
SNX	0.393	2.034	88	0.228^{b}	1.770	80
SLX	0.548	1.716	93	0.262^{ab}	2.042	73
SMX	0.264	1.853	89	0.277^{ab}	2.097	80
SHX	0.456	2.192	104	0.320^{a}	2.080	73
SEM	0.052	0.079	4	0.011	0.076	2
P-value	0.389	0.158	0.362	0.017	0.394	0.306

[1]Data are presented as the mean value of 10 birds per replicate cage (n = 6 replicate cages). SNX = non-oxidized soybean oil (fresh soybean oil); SLX = lowly-oxidized soybean oil (heated for 10 h); SMX = moderately-oxidized soybean oil (heated for 18 h); SHX = highly-oxidized soybean oil (heated for 30 h). MDA = malonaldehyde; T-AOC = total antioxidant capacity; T-SOD = total superoxide dismutase. mg prot =milligram protein. SEM = standard error of the mean.

[2]Soybean oils were included in the diet at a level of 4%.

[a,b]Means in the same column with different superscript letters are significantly different (p<0.05).

The activities of T-SOD in the ileal mucosa both on days 14 and 21 were affected (p<0.05) by dietary oxidized oil treatment (Table 7). Chickens fed moderately- and highly-oxidized soybean oil presented lower (p<0.05) T-SOD activity in the ileal mucosa compared with those fed non-oxidized soybean oil diet. The different oxidation levels of soybean oils did not affect MDA concentrations on days 14 and 21 or T-AOC activity in the ileal mucosa on days 14 and 21 (p>0.05).

Intestinal morphology

The intestinal morphology of the jejunum and ileum were not significantly affected (p>0.05, Table 8 and 9) by dietaryoxidized-oil treatments.

Claudin-1, Occludin, TNF-α, IL-22, and CAT mRNA Expression in the ileum

The mRNA expression of claudin-1 in the ileum tended (p=0.056) to be down regulated by the dietary addition of oxidized oils (Figure1). The mRNA

Table 7 – Levels of antioxidant-related compounds in the ileal mucosa of broilers fed diets with fresh or oxidized soybean oils[1, 2].

Treatment	14d			21d		
	MDA(nmol/ mg prot)	T-AOC(U /mg prot)	T-SOD(U /mg prot)	MDA(nmol/ mg prot)	T-AOC(U /mg prot)	T-SOD(U /mg prot)
SNX	0.206	1.351	87[a]	0.202	1.756	92[a]
SLX	0.199	1.280	82[a]	0.191	1.712	83[ab]
SMX	0.165	1.280	77[ab]	0.188	1.654	77[b]
SHX	0.150	1.404	72[b]	0.230	1.594	74[b]
SEM	0.011	0.028	2	0.024	0.043	2
p-value	0.208	0.341	0.025	0.160	0.603	0.013

[1]Data are presented as the mean value of 10 birds per replicate cage (n = 6 replicate cages). SNX = non-oxidized soybean oil (fresh soybean oil); SLX = lowly-oxidized soybean oil (heated for 10 h); SMX = moderately-oxidized soybean oil (heated for 18 h); SHX = highly-oxidized soybean oil (heated for 30 h). MDA = malonaldehyde; T-AOC = total antioxidant capacity; T-SOD = total superoxide dismutase. mg prot =milligram protein. SEM = standard error of the mean.

[2]Soybean oils were included in the diet at a level of 4%.

[a,b]Means in the same column with different superscript letters are significantly different (p<0.05).

Table 8 – Intestinal morphology in the jejunum mucosa of broilers fed diets with fresh or oxidized soybean oils[1, 2].

Treatment	14d			21d		
	Villus height (um)	Crypt depth (um)	Villus height/Crypt depth	Villus height (um)	Crypt depth (um)	Villus height/Crypt depth
SNX	668	211	3.304	865	247	3.495
SLX	684	216	3.359	812	237	3.743
SMX	689	203	3.263	918	227	4.307
SHX	737	221	3.616	775	270	2.764
SEM	13.389	8.502	0.095	23.499	10.637	0.212
P-value	0.320	0.989	0.490	0.145	0.859	0.080

[1]Data are presented as the mean value of 10 birds per replicate cage (n = 6 replicate cages). SNX = non-oxidized soybean oil (fresh soybean oil); SLX = lowly-oxidized soybean oil (heated for 10 h); SMX = moderately-oxidized soybean oil (heated for 18 h); SHX = highly-oxidized soybean oil (heated for 30 h). SEM = standard error of the mean.

[2]Soybean oils were included in the diet at a level of 4%.

[a, b] Means in the same column with different superscript letters are significantly different (p<0.05).

Table 9 – Intestinal morphology in the ileal mucosa of broilers fed diets with fresh and oxidized soybean oils[1, 2].

Treatment	14d			21d		
	Villus height (um)	Crypt depth (um)	Villus height/Crypt depth	Villus height (um)	Crypt depth (um)	Villus height/Crypt depth
SNX	549	158	3.598	737	211	3.695
SLX	543	175	3.627	633	177	3.738
SMX	533	169	3.263	664	193	3.687
SHX	508	144	3.616	632	185	3.540
SEM	23.000	4.867	0.095	19.818	7.469	0.096
p-value	0.686	0.114	0.490	0.182	0.442	0.909

[1]Data are presented as the mean value of 10 birds per replicate cage (n = 6 replicate cages). SNX = non-oxidized soybean oil (fresh soybean oil); SLX = lowly-oxidized soybean oil (heated for 10 h); SMX = moderately-oxidized soybean oil (heated for 18 h); SHX = highly-oxidized soybean oil (heated for 30 h).

[2]Soybean oils were included in the diet at a level of 4%

[a, b] Means in the same column with different superscript letters are significantly different (p<0.05).

expression of occludin, IL-22, and CAT in ileum were affected by dietary oxidized oil treatment (p<0.05). Chickens fed moderately- and highly-oxidized soybean oil had higher (p<0.05) mRNA expression of IL-22, but lower (p<0.05) mRNA expression of occludin and CAT compared with those fed the non-oxidized soybean oil diet. The mRNA expression of CAT was down regulated by oxidized soybean oil treatments (p<0.05).

DISCUSSION

The impact of dietary oxidized oil on growth had been observed in broiler chicks (Ehr *et al.*, 2015), weaned pigs (Li *et al.*, 2012), and different aquatic animals (Lewis-McCrea & Lall, 2007; Dong *et al.*, 2011). Some studies reported that dietary oxidized oils impaired animal performance (Tavarez *et al.*, 2011, Wang *et al.*, 2015), while others did not find

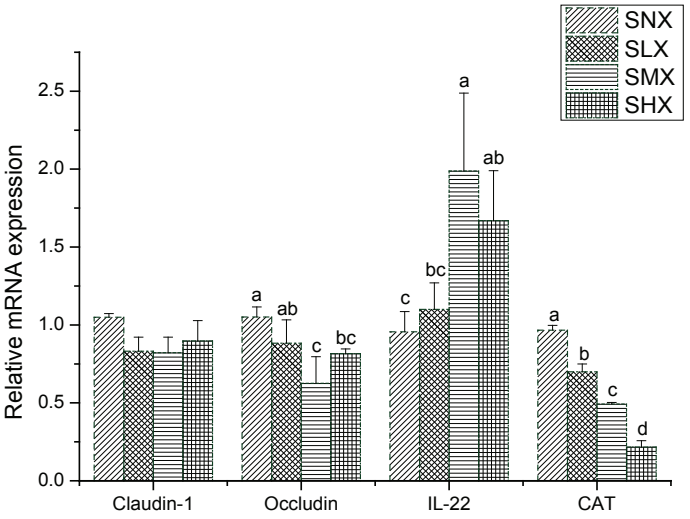

Figure 1 – Relative mRNA expression levels of Claudin-1, Occludin, IL-22, and CAT. Data are mean values of 6 individual birds (1 per replicate cage). SNX = birds fed non--oxidized soybean oil; SLX = birds fed lowly-oxidized soybean oil; SMX = birds fed moderately-oxidized soybean oil; SHX = birds fed highly-oxidized soybean oil. β-actin was used as an endogenous reference gene, and mRNA expression is expressed relative value to the SNX group. IL-22 = interleukin; CAT = catalase. SEM = standard error of the mean. Soybean oils were included in the diet at a level of 4%.a, b, c and d: means in the same column with different superscript letters are significantly different (p<0.05).

any negative effects (Dong *et al.*, 2011). This may be related to differences in dietary oil content and degree of oil oxidation (Yue *et al.*, 2010, Zhang *et al.*, 2011). In our study, no significant effects of feeding diets with different oxidation levels of soybean oils on broiler growth performance were detected, possibly due to the low oil content of the experimental diets and their low oxidation levels.

The primary physiological response of poultry when the body suffers oxidative stress is to activate the hypothalamic-pituitary-adrenal (HPA) axis. The response is characterized by adrenal cortical hypertrophy and increased synthesis and release of adrenal glucocorticoids, primarily corticosterone (Zhang *et al.*, 2009). The increase in corticosterone levels by feeding oxidized oils in current study indicated that the broilers were under oxidative stress.

MDA is a lipid peroxidation marker, and increasing levels are related to lipid peroxidation and oxidation stress (Aytekin *et al.*, 2015, Esgalhado *et al.*, 2015). Our data showed that the dietary inclusion of highly-oxidized soybean oil increased the concentration of MDA in the jejunum and the liver, indicating lipid peroxidation in these tissues. Similar results were obtained in pigs (Ringseis *et al.*, 2007) and broilers (Zhang et al., 2011; Liang et al., 2015). MDA level changes observed in the birds fed oxidized oils maybe triggered by free radicals of lipid peroxidation.

Other biomarkers of metabolic oxidative status are total antioxidant capacity (T-AOC) levels, and total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-PX) activities. Liver T-AOC level on day 21 was reduced in SMX- and SHX-fed broilers compared with those fed SNX, but no changes in these parameters at other evaluated time points or tissues. In addition, liver T-AOC activity of SMX and SHX diet treatments was reduced between d 14 and d 21, which may suggest that their liver suffered oxidative stress (Gao *et al.*, 2013, Zhang *et al.*, 2015). Total superoxide dismutases (T-SOD) are important antioxidant enzymes that accept an electron from superoxide anion (O^{2-}) and H_2O to generate hydrogen peroxide (H_2O_2) (Tan *et al.*, 2015). Glutathione peroxidase (GHS-Px) may prevent cell membrane oxidative damage caused by lipid peroxides (LOOH). In the present study, T-SOD levels both inileum (d 14 and 21) and liver (d 21) and the expression of CAT gene in ileum significantly decreased when oxidized oils were fed. These results were consistent with those reported in another oxidative stress study (Liang *et al.*, 2015). The present study demonstrated that feeding oxidized oil decreased the activity of antioxidation enzymes (T-SOD), indicating the lower capacity of scavenging free radicals, and suggested that the oxidized oil might cause metabolic oxidative stress in intestine of broiler chickens.

Claudins and occludin play a critical roles in the intestinal barrier function and paracellular permeable selectivity in the intestinal tissue (Cani *et al.*, 2008; Lee 2015). Claudins, including claudin-1, -3, -5, -11, and -19, have been associated with a more general barrier-tightening function (Overgaard *et al.*, 2011). Occludin influences the tight junction structure and permeability of the intestinal epithelia (Al-Sadi *et al.*, 2011). Disruption of the tight junctions increases intestinal permeability. The mRNA expression of occludin and claudin-1 in intestines of broilers were detected by real-time PCR. The results showed that feeding oxidized oils down regulated occludin and claudin-1 mRNA expression in the ileum and suggest that the regulation of occludin and claudin-1 expression by oxidized oils may be increase intestinal permeability, leading intestinal barrier dysfunction. These results were in agreement with other reports on animals suffering oxidative stress (Casselbrant *et al.*, 2015, Nevado *et al.*, 2015, Suzuki & Hara, 2010). According to John et al. (2011), excessive levels of reactive oxygen species in intestines may contribute to barrier dysfunction, as indicated by a disruption of composition of the tight junctions.

Interleukin-22 (IL-22) is produced by activated T cells and signals through a receptor complex consisting of IL-22R1 and IL-10R2 (Brand *et al.*, 2006). In present study, the IL-22 mRNA expression in the ileum was upregulated in SMX- and SHX-fed broilers, indicating feeding oxidized oils can induce inflammation through the upregulation of IL-22 mRNA expression.

In conclusion, feeding oxidized soybean oil impaired the intestinal barrier function and induced metabolic oxidative status of broiler chickens. The results suggested that the inclusion of oxidized soybean oil in broiler feeds may induce oxidative stress in intestines, resulting in intestinal barrier dysfunction, which may be partially related to an increase in cytokine levels.

ACKNOWLEDGEMENTS

The authors thank Yuxin Shao, Yanyan Yang, Yuanyang Dong, Xuan Liu and He Gao for their help with the experiments. This work was supported by the Yangtz River Scholar and Innovation Research Team Development Program (No. IRT0945), the Beijing Higher Education Young Elite Teacher Project, and the Chinese Universities Scientific Fund (No. 2015DK005).

REFERENCES

Al-Orf SM. Effect of oxidized phosphatidylcholine on biomarkers of oxidative stress in rats. Indian Journal of Clinical Biochemistry 2011;26:154-160.

Al-Sadi R, Khatib K, Guo S, Ye D, Youssef M, Ma T. Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier. American Journal of Physiology Gastrointestand Liver Physiology 2011;300:G1054-1064.

Awada M, Soulage CO, Meynier A, Debard C, Plaisancie P, Benoit B, et al. Dietary oxidized n-3 PUFA induce oxidative stress and inflammation: role of intestinal absorption of 4-HHE and reactivity in intestinal cells. Journal of Lipid Research 2012;53:2069-2080.

Aytekin I, Aksit H, Sait A, Kaya F, Aksit D, Gokmen Mand Baca AU. Evaluation of oxidative stress via total antioxidant status, sialic acid, malondialdehyde and RT-PCR findings in sheep affected with bluetongue. Veterinary Record Open 2015;2:e000054-e000061.

Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, et al . IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. American Journal of Physiology-Gastrointestinal Liver Physiology 2006;290:G827-838.

Cabuk H, Kokturk M. Low density solvent-based dispersive liquid-liquid microextraction for the determination of synthetic antioxidants in beverages by high-performance liquid chromatography. Scientific World Journal 2013;2013: 398-414.

Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 2008;57:1470-1481.

Casselbrant A, Elias E, Fandriks L, Wallenius V. Expression of tight-junction proteins in human proximal small intestinal mucosa before and after Roux-en-Y gastric bypass surgery. Surgeryfor Obesity and Related Diseases 2015;11:45-53.

Choe E, Min DB. Chemistry of deep-fat frying oils. Journal of Food Science 2007;72:R77-86.

Dibner JJ, CA A, Kitchell ML, Shermer WD, Ivey FJ. Feeding of oxidized fats to broilers and swine: effects on enterocyte turnover, hepatocyte proliferation and the gut associated lymphoid tissue. Animal Feed Science Technology 1996;62:1-13.

Dong XL, Lei W, Zhu XM, Han D, Yang YX, Xie SQ. Effects of dietary oxidized fish oil on growth performance and skin colour of Chinese longsnout catfish (Leiocassis longirostris Günther). Aquaculture Nutrition 2011;17:e861-e868.

Ehr IJ, Kerr BJ, Persia ME. Effects of peroxidized corn oil on performance, AMEn, and abdominal fat pad weight in broiler chicks. Poultry Science 2015;94:1629-1634.

El-Bahr SM. Effect of curcumin on hepatic antioxidant enzymes activities and gene expressions in rats intoxicated with aflatoxin B1. Phytotherapy Research 2015;29:134-140.

Esgalhado M, Stockler-Pinto MB, Franca Cardozo de LF, Costa C, Barboza JE, Mafra D. Effect of acute intradialytic strength physical exercise on oxidative stress and inflammatory responses in hemodialysis patients. Kidney Research and Clinical Practice 2015;34:35-40.

Fontagné S, Bazin D, Brèque J, Vachot C, Bernarde C, Rouault T, Bergot P. Effects of dietary oxidized lipid and vitamin A on the early development and antioxidant status of Siberian sturgeon (Acipenserbaeri) larvae. Aquaculture 2006;257:400-411.

Gao YY, Xie QM, Ma JY, Zhang XB, Zhu JM, Shu DM, et al. Supplementation of xanthophylls increased antioxidant capacity and decreased lipid peroxidation in hens and chicks. British Journal of Nutrition 2013;109:977-983.

Hayam I, Cogan U, Mokady S. Enhanced peroxidation of proteins of the erythrocyte membrane and of muscle tissue by dietary oxidized oil. Bioscience Biotechnology and Biochemistry 1997;61:1011-1012.

ISO - International Organization for Standarization. Animal and vegetable fats and oils. Determination of peroxide value [ISO 3960:2001 IDT]. Geneva; 2001.

ISO - International Organization for Standarization. Animal and vegetable fats and oils. Determination of anisidine value [ISO 6885:2006 IDT]. Geneva; 2006.

John LJ, Fromm M, Schulzke JD. Epithelial barriers in intestinal inflammation. Antioxidants & Redox Signaling 2011;15:1255-1270.

Kumagai T, Matsukawa N, Kaneko Y, Kusumi Y, Mitsumata M, Uchida K. A lipid peroxidation-derived inflammatory mediator: identification of 4-hydroxy-2-nonenal as a potential inducer of cyclooxygenase-2 in macrophages. Journal of Biological Chemistry 2004;279:48389-48396.

Lee SH. Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. Intestinal Research 2015;13:11-18.

Lewis-McCrea LM, Lall SP. Effects of moderately oxidized dietary lipid and the role of vitamin E on the development of skeletal abnormalities in juvenile Atlantic halibut (Hippoglossus hippoglossus). Aquaculture Nutrition 2007;262:142-155.

Li P, Piao X, Ru Y, Han X, Xue L, Zhang H. Effects of adding essential oil to the diet of weaned pigs on performance, nutrient utilization, immune response and intestinal health. Asian-Australasian Journal of Animal Sciences 2012;25:1617-1626.

Liang F, Jiang S, Mo Y, Zhou G,Yang L. Consumption of oxidized soybean oil increased intestinal oxidative stress and affected intestinal immune variables in yellow-feathered broilers. Asian-Australasian Journal of Animal Sciences 2015;28:1194-1201.

Liu P, Kerr BJ, Weber TE, Chen C, Johnston LJ, Shurson GC. Influence of thermally oxidized vegetable oils and animal fats on intestinal barrier function and immune variables in young pigs. Journal of Animal Science 2014;92:2971-2979.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402-408.

Mujahid A, Akiba Y, Toyomizu M. Olive oil-supplemented diet alleviates acute heat stress-induced mitochondrial ROS production in chicken skeletal muscle. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 2009;297:R690-698.

Nevado R, Forcén R, Layunta E, Murillo MD, Grasa L. Neomycin and bacitracin reduce the intestinal permeability in mice and increase the expression of some tight-junction proteins. Revista Española de Enfermedades Digestivas 2015;107:1-14.

Overgaard CE, Daugherty BL, Mitchell LA, Koval M. Claudins: control of barrier function and regulation in response to oxidant stress. Antioxidations and Redox Signaling 2011;15:1179-1193.

Paul S, Mittal GS. Regulating the use of degraded oil/fat in deep-fat/oil food frying. Critical Reviews in Food Science Nutrition 1997;37:635-662.

Poulli KI, Mousdis GA, Georgiou CA. Monitoring olive oil oxidation under thermal and UV stress through synchronous fluorescence spectroscopy and classical assays. Food Chemistry 2009;117:499-503.

Ringseis R, Gutgesell A, Dathe C, Brandsch C, Eder K. Feeding oxidized fat during pregnancy up-regulates expression of PPARalpha-responsive genes in the liver of rat fetuses. Lipids in Health and Disease 2007;6:1-13.

Sidwell CG, Milada Benca HS, Mttchel JR JH. The Use of Thiobarbituric Acid as a Measure of Fat Oxidation. The Journal of the American Oil Chemist's Society 1953;31:603-606.

Smyk B. Singlet oxygen autoxidation of vegetable oils: evidences for lack of synergy between beta-carotene and tocopherols. Food Chemistry 2015;182:209-216.

Suzuki T, Hara H. Dietary fat and bile juice, but not obesity, are responsible for the increase in small intestinal permeability induced through the suppression of tight junction protein expression in LETO and OLETF rats. Nutrition and Metabolism 2010;7:1-19.

Tan LG, Xiao JH, Yu DL, Zhang L, Zheng F, Guo LY, et al. PEP-1-SOD1 fusion proteins block cardiac myofibroblast activation and angiotensin II-induced collagen production. BMC Cardiovascular Disorders 2015;15:116-125.

Tavarez MA, Boler DD, Bess KN, Zhao J, Yan F, Dilger AC, et al. Effect of antioxidant inclusion and oil quality on broiler performance, meat quality, and lipid oxidation. Poultry Science 2011;90:922-930.

Totani N, Burenjargal M, Yawata MandOjiri Y. Chemical properties and cytotoxicity of thermally oxidized oil. Journal of Oleo Science 2008;57:153-160.

Totani N, Yawata M, Takada M, Moriya M. Acrylamide content of commercial frying oil. Journal of Oleo Science 2007;56:103-106.

Wang LG, Li EC, Qin JG, Du ZY, Yu N, et al. Effect of oxidized fish oil and α-tocopherol on growth, antioxidation status, serum immune enzyme activity and resistance to Aeromonas hydrophila challenge of Chinese mitten crab Eriocheir sinensis. Aquaculture Nutrition 2015;21:414-424.

Yang SP, Liu HL, Wang CG, Yang P, Sun CB, Chan SM. Effect of oxidized fish oil on growth performance and oxidative stress of Litopenaeus vannamei. Aquaculture Nutrition 2015;21:121-127.

Yue HY, Wang J, Qi XL, Ji F, Liu MF, Wu SG, et al. Effects of dietary oxidized oil on laying performance, lipid metabolism, and a polipoprotein gene expression in laying hens. Poultry Science 2011;90:1728-1736.

Yue HY, Zhang L, Wu SG, Xu L, Zhang HJ, Qi GH. Effects of transport stress on blood metabolism, glycolytic potential, and meat quality in meat-type yellow-feathered chickens. Poultry Science 2010;89:413-419.

Zhang L, Yue HY, Zhang HJ, Xu L, Wu SG, Yan HJ, et al. Transport stress in broilers: I. Blood metabolism, glycolytic potential, and meat quality. Poultry Science 2009;88:2033-2041.

Zhang Q, Saleh AS, Chen J, Shen Q. Chemical alterations taken place during deep-fat frying based on certain reaction products: a review. Chemistry and Physics of Lipids 2012;165:662-681.

Zhang W, Xiao S, Lee EJ, Ahn DU. Consumption of oxidized oil increases oxidative stress in broilers and affects the quality of breast meat. Journal of Agricultural and Food Chemistry 2011a;59:969-974.

Zhang WH, Gao F, Zhu QF, Li C, Jiang Y, et al. Dietary sodium butyrate alleviates the oxidative stress induced by corticosterone exposure and improves meat quality in broiler chickens. Poultry Science 2011;90:2592-2599.

Zhang XH, Sun ZY, Cao FL, Ahmad H, Yang XH, et al. Effects of dietary supplementation with fermented ginkgo leaves on antioxidant capacity, intestinal morphology and microbial ecology in broiler chicks. British Poultry Science 2015;56:370-380.