



## Effects of soybean isoflavone on intestinal antioxidant capacity and cytokines in young piglets fed oxidized fish oil\*

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**Abstract:** To investigate the effect of glycitein, a synthetic soybean isoflavone (ISF), on the intestinal antioxidant capacity, morphology, and cytokine content in young piglets fed oxidized fish oil, 72 4-d-old male piglets were assigned to three treatments. The control group was fed a basal diet containing fresh fish oil, and the other two groups received the same diet except for the substitution with the same dosage of oxidized fish oil alone or with ISF (oxidized fish oil plus ISF). After 21 d of feeding, supplementation of oxidized fish oil increased the levels of malondialdehyde (MDA), oxidized glutathione (GSSG), interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-2 (IL-2), nuclear factor  $\kappa$  B (NF- $\kappa$ B), inducible nitric oxide synthase (iNOS), NO, and Caspase-3 in jejunal mucosa, and decreased the villous height in duodenum and the levels of secretory immunoglobulin A (sIgA) and IL-4 in the jejunal mucosa compared with supplementation with fresh oil. The addition of oxidized fish oil plus ISF partially alleviated this negative effect. The addition of oxidized fish oil plus ISF increased the villous height and levels of sIgA and IL-4 in jejunal mucosa, but decreased the levels of IL-1 $\beta$  and IL-2 in jejunal mucosa ( $P < 0.05$ ) compared with oxidized fish oil. Collectively, these results show that dietary supplementation of ISF could partly alleviate the negative effect of oxidized fish oil by improving the intestinal morphology as well as the antioxidant capacity and immune function in young piglets.

**Key words:** Young piglets, Oxidized fish oil, Growth performance, Soybean isoflavone, Antioxidant capacity  
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### 1 Introduction

The intestine is an important organ in the defense against harmful endogenous and exogenous agents (Blikslager *et al.*, 2007). However, many factors such

as pathogens, polyunsaturated fatty acids, and mildew or oxidized feed can induce the oxidative stress (Slater, 1984; Halliwell and Chirico, 1993; Moreto and Perez-Bosque, 2009), especially in neonatal piglets (Chamulitrat *et al.*, 1996; Aw, 1999; Lackeyram *et al.*, 2012). These “stressors” induce intestinal injury and dysfunction by producing excessive reactive oxygen species (ROS) and destroy the antioxidant capacity in neonatal piglets (Moreto and Perez-Bosque, 2009; Maeda *et al.*, 2010). Also, a series of pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukins-1 $\alpha$ , -1 $\beta$ , -6 (IL-1 $\alpha$ , IL-1 $\beta$ , IL-6) also are produced (Kelley, 2001). Oxidative stress involving ROS production is an

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important mediator of inflammation and immune activation (Aw, 1999; Ypsilantis *et al.*, 2008), and ROS can cause injury to the intestinal mucosa (Mercer *et al.*, 1996), but the mechanisms are not understood completely. The present study was designed to explore intestinal oxidative stress, provoked by including oxidized fish oil in the diet of young piglets, in an attempt to explain its influence on the development and immune function of the intestinal mucosa. Oxidative stress is lessened by the antioxidants (Otani *et al.*, 2000; Kelley, 2001). Accumulating evidence suggests that supplementation of additives with antioxidant function (such as vitamin E, selenium, glutamine, and arginine) to the feed of piglets (Ban *et al.*, 2004; Huang *et al.*, 2011; Xi *et al.*, 2011; Zhan *et al.*, 2011) can reduce the oxidative stress and protect the intestinal function. Soybean isoflavones (ISFs) are stronger antioxidants, and have been studied extensively (Yousef *et al.*, 2004; Foti *et al.*, 2005; Lee *et al.*, 2005; Jiang *et al.*, 2007; Dixit *et al.*, 2012). ISFs may inhibit the production and enhance the clearance of free radicals by inducing endogenous antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), etc. These results led us to speculate that dietary ISFs may reduce the intestinal oxidative stress in young piglets. The potential protective effects of ISFs on the intestinal oxidative stress in very young piglets have not been reported. The objective of the present study was to elucidate whether and how dietary ISFs affect the growth performance, intestinal antioxidant capacity, and intestinal function in young piglets fed oxidized fish oil.

## 2 Materials and methods

### 2.1 Animals and diets

Seventy-two 4-d-old male piglets Duroc×(Landrace×Yorkshire) were weighed ((1.80±0.04) kg) and randomly allotted to three treatments, each with six replicates of four animals. Each replicate of piglets was individually housed in 1.5 m×1.0 m pens with slatted stainless steel floors in a room held at (32±1) °C with constant humidity. Piglets were fed a diet formulated to meet the requirements of NRC (2012) recommendation for 3–5 kg piglets (Table 1). The lipid source for the control group was 50 g/kg

fresh fish oil (Beihan Evergreen Aquatic Product Science & Technology Co. Ltd., Guangdong, China), and the other two groups received the same diet except for the substitution with 50 g/kg oxidized fish oil or 50 g/kg oxidized fish oil plus 20 mg/kg ISF (prepared at the Institute of Animal Science, Guangdong Academy of Agricultural Sciences, containing 98.4% glycitein). The fish oil was oxidized, as described by Andrew *et al.* (1960), and then stored at –20 °C. The peroxide values were 186.89 mmol/kg for the oxidized fish oil and 4.20 mmol/kg for fresh fish oil. Details of ingredient composition and the calculated nutrient content of the diet are provided in Table 1. The diets were mixed with four weight equivalents of water just before each feeding and were provided without restriction every 3 h from 06:00 to 24:00. Piglets had free access to water. The animals quickly became accustomed to the feeding method and

**Table 1 Ingredients and composition of the basal diet for young piglets (dry matter content)**

Ingredient	Composition (%)
Whole milk powder	48.00
Whey protein concentrate	20.00
Plasma protein powder	5.00
Fresh fish oil/oxidized fish oil	5.00
Whey	6.89
D,L-Methionine (98%)	0.30
L-Tryptophan (98%)	0.02
Liquid choline chloride (70%)	0.12
Premix <sup>1,2,3</sup>	0.82
Total	100.00
Chemical composition	
CP	24.94
ME (MJ/kg)	18.16
Crude fat	15.16
Lactose	26.59
Lys	2.07
Met+Cys	1.20
Thr	1.20
Trp	1.20
Ca	1.02
EP	0.59

<sup>1</sup> Premix was composed as follows (g/kg premix): piglet multi-vitamins 0.50 g, minerals 2.00 g, and emulsifying agent 1.00 g.

<sup>2</sup> Vitamins contain: retinyl palmitate 6600 IU, cholecalciferol 660 IU, all-rac- $\alpha$ -tocopheryl acetate 48 IU, menadione sodium bisulfite 1.50 mg, biotin 0.24 mg, folic acid 0.90 mg, nicotinic acid 60.0 mg, calcium pantothenate 36 mg, riboflavin 12.0 mg, thiamin 4.5 mg, pyridoxine 6.0 mg, cyanocobalamin 60.0  $\mu$ g. <sup>3</sup> Minerals contain: ferric 105 mg, copper 10 mg, manganese 5 mg, zinc 110 mg. CP: crude protein; ME: metabolizable energy; EP: essential fat

consumed each meal without spillage. Feed intake (on an as-fed basis) per pen was calculated based on 25% of the weight difference of the feed before and after each feeding (dry weight/liquid weight=1:4). The body weights (BW) of all piglets were measured on the 1st day and the 21st day of the experiment. Average daily gain (ADG), average daily feed intake (ADFI, dry weight), and gain/feed (G:F) ratios were calculated.

## 2.2 Sample collection

Two randomly chosen piglets from each pen were fasted for 12 h and then sacrificed after weighing on the 21st day of experiment by muscular injection of sodium pentobarbital (40 mg/kg of BW). The small intestine, immediately ligated from the pyloric sphincter to the ileocolonic junction, was rapidly removed along the mesenteric border on an ice-cold metal plate. The segment of small intestine proximal to the ligament of Treitz was designated as the duodenum; the remainder was divided into two equal lengths, jejunum proximal and ileum distal, as described by Burrin *et al.* (2000). A 5-cm piece from the middle of the jejunum was opened longitudinally and gently but thoroughly cleaned with ice-cold phosphate buffered saline (PBS) containing 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, and 2 mmol/L KH<sub>2</sub>PO<sub>4</sub>, pH 7.4. Jejunal mucosa was collected by scraping with a glass slide, and a portion was snap-frozen in liquid nitrogen.

## 2.3 Small intestinal morphology

Additional samples were collected from consistent locations (the duodenal sampling site was located 2 cm from the opening of the pancreatic duct, and the ileum sampling site was located 2 cm from the cecum) of each segment of the intestine, rinsed as above, and then fixed in 10% neutral buffered formalin overnight or longer. Paraffin-embedded sections were prepared from each sample in duplicate, stained with hematoxylin and eosin, and used for histometric measurements, as described by Jensen *et al.* (2001). The mean villous height, crypt depth, and intestinal circumference were measured using an Axiophot microscope (Philips, Holland) and National Institutes of Health (NIH) image software version 1.60 (Bethesda, MD, USA) in at least 20 vertically well-oriented villus-crypt columns. The villous area was calculated as described by Frankel *et al.* (1993).

## 2.4 Analyses of antioxidant enzymes and cytokines

The frozen jejunal mucosa was homogenized in PBS (9 ml added per gram tissue) at 4 °C using an Ultra-Turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany) for 1–2 min at 3000–5000 r/min. The homogenates were centrifuged (4000g for 5 min at 4 °C) and the supernatants were used for determining the indices of antioxidant capacity and concentrations of cytokines. The antioxidant indicators, namely GSH-Px, SOD, CAT, GSH, oxidized glutathione (GSSG), and malondialdehyde (MDA), were quantified using assay kits (Nanjing Jiancheng Bio-engineering Institute, China), previously validated in house for porcine tissue. Secretory immunoglobulin A (sIgA), IL-1 $\beta$ , IL-2, IL-4, IL-10, interferon- $\gamma$  (IFN- $\gamma$ ), TNF- $\alpha$ , inducible nitric oxide synthase (iNOS), and nuclear factor  $\kappa$  B (NF- $\kappa$ B) were measured using porcine enzyme-linked immunosorbent assay (ELISA) kits (Groundwork Biotechnology Diagnosticate, San Diego, CA, USA) through an automated ELISA reader (Model 550, Bio-Rad, Hercules, CA, USA).

## 2.5 Statistical analysis

All data were analyzed using replicates as the experimental unit ( $n=6$ ). The effect of treatment on each variable was analyzed by one-way analysis of variance (ANOVA) using the GLM procedure of SAS (Version 8.2, SAS Institute, Cary, NC, USA).  $P \leq 0.05$  was considered to be statistically different.

## 3 Results

### 3.1 Growth performance

The effects of feeding ISF and oxidized fish oil for 21 d on growth performance of young piglets are shown in Table 2. The final BW, ADG, or G:F ratio of piglets fed oxidized fish oil was significantly lower than that of piglets in control group ( $P < 0.05$ ), while the final BW, ADG, or G:F ratio of piglets fed oxidized fish oil plus ISF has a trend to increase compared with those piglets fed oxidized fish oil. There was no difference in ADFI among treatments ( $P > 0.05$ ).

### 3.2 Small intestinal morphology

The piglets fed oxidized fish oil had significantly lower villous height in the duodenum, jejunum, and

ileum than the piglets fed fresh fish oil and those fed oxidized fish oil plus ISF ( $P<0.05$ ; Table 3). Crypt depth, villous height/crypt depth, and villous area in the jejunum were not significantly different among the three treatments ( $P>0.05$ ).

### 3.3 Antioxidant capacity in jejunal mucosa

The effects of ISF and oxidized fish oil for 21 d of feeding on the antioxidant capacity in the jejunal mucosa of neonatal piglets are shown in Table 4. The levels of MDA and GSSG in piglets fed fresh fish oil

diet and oxidized fish oil plus ISF diets were all significantly lower than those in piglets fed oxidized fish oil diet alone ( $P<0.05$ ). The ratio of GSH to GSSG was higher in piglets fed fresh fish oil diets than in those fed oxidized fish oil alone or oxidized fish oil plus ISF ( $P<0.05$ ). The activity of GSH-Px in piglets fed oxidized fish oil plus ISF was significantly higher than that in piglets fed oxidized fish oil alone or fresh fish oil alone. There were no differences in SOD, CAT, or GSH among three treatments ( $P>0.05$ ).

**Table 2** Effects of dietary soybean isoflavone addition on growth performance of young piglets fed a diet with oxidized fish oil

Treatment <sup>1</sup>	Initial BW (kg)	Final BW (kg)	ADG (g/d)	ADFI (g/d)	G/F (g/g)
FFO	1.77	7.58 <sup>a</sup>	290.00 <sup>a</sup>	240.00	1.20 <sup>a</sup>
OFO	1.78	6.70 <sup>b</sup>	255.00 <sup>b</sup>	239.00	1.06 <sup>b</sup>
OFO+20 ISF	1.79	7.15 <sup>ab</sup>	268.00 <sup>ab</sup>	240.00	1.11 <sup>ab</sup>
SEM	0.04	0.18	7.73	4.95	0.03
<i>P</i> -value	0.947	0.047	0.019	0.955	0.038

<sup>a, b</sup> Means ( $n=6$ ) in a column with different superscripts are significantly different ( $P<0.05$ ). <sup>1</sup> Diets are designated as FFO for fresh fish oil diet or OFO for oxidized fish oil diet; 20 ISF is supplemented with 20 mg/kg soybean isoflavone. BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; G/F: gain/feed; SEM: standard error of the mean

**Table 3** Effect of dietary soybean isoflavone addition on morphology of intestine in young piglets fed a diet with oxidized fish oil

Treatment <sup>1</sup>	Villous height ( $\mu\text{m}$ )			Crypt depth ( $\mu\text{m}$ )		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
FFO	502.00 <sup>a</sup>	536.00 <sup>a</sup>	495.00 <sup>a</sup>	169.00	144.00	148.00
OFO	439.00 <sup>b</sup>	465.00 <sup>b</sup>	478.00 <sup>b</sup>	150.00	183.00	177.00
OFO+20 ISF	539.00 <sup>a</sup>	503.00 <sup>a</sup>	498.00 <sup>a</sup>	199.00	153.00	139.00
SEM	18.74	20.61	22.14	15.46	18.65	16.72
<i>P</i> -value	0.009	0.047	0.050	0.116	0.313	0.280
Treatment <sup>1</sup>	Villous height: crypt depth			Villous area ( $\times 10^3 \mu\text{m}^2$ )		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
FFO	3.09	3.65	2.83	3.48	3.71	3.18
OFO	3.00	3.00	3.30	3.20	3.13	2.58
OFO+20 ISF	2.56	3.31	3.45	4.14	3.70	3.40
SEM	0.19	0.31	0.30	0.36	0.24	0.41
<i>P</i> -value	0.196	0.347	0.431	0.201	0.171	0.471

<sup>a, b</sup> Means ( $n=6$ ) in a column with different superscripts are significantly different ( $P<0.05$ ). <sup>1</sup> Diets are designated as FFO for fresh fish oil diet or OFO for oxidized fish oil diet; 20 ISF is supplemented with 20 mg/kg soybean isoflavone; SEM: standard error of the mean

**Table 4** Effect of dietary soybean isoflavone addition on antioxidant indices of jejunal mucosa in young piglets fed a diet with oxidized fish oil

Treatment <sup>1</sup>	MDA (mmol/ml)	SOD (U/g prot)	GSH-Px (U/g prot)	CAT (U/g prot)	GSH (mmol/mg prot)	GSSG (mmol/mg prot)	GSH/GSSG
FFO	3.17 <sup>b</sup>	9.14	53.33 <sup>b</sup>	1.32	10.88	8.58 <sup>b</sup>	1.35 <sup>a</sup>
OFO	5.20 <sup>a</sup>	7.21	59.84 <sup>b</sup>	1.23	9.08	12.62 <sup>a</sup>	0.77 <sup>b</sup>
OFO+20 ISF	2.40 <sup>c</sup>	8.33	91.46 <sup>a</sup>	1.80	8.33	9.56 <sup>b</sup>	0.89 <sup>b</sup>
SEM	0.03	0.68	7.29	0.17	1.01	1.09	0.15
<i>P</i> -value	<0.001	0.147	0.005	0.073	0.219	0.048	0.031

<sup>a, b, c</sup> Means ( $n=6$ ) in a column with different superscripts are significantly different ( $P<0.05$ ). <sup>1</sup> Diets are designated as FFO for fresh fish oil diet or OFO for oxidized fish oil diet; 20 ISF is supplemented with 20 mg/kg soybean isoflavone. MDA: maleic dialdehyde; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; CAT: catalase; GSH: glutathione; GSSG: oxidized glutathione; prot: protein; SEM: standard error of the mean

### 3.4 Cell-mediated immunologic factors

Piglets fed oxidized fish oil diet had lower concentrations of sIgA and higher IL-1 $\beta$  and IL-2 in jejunal mucosa than piglets fed fresh fish oil and those fed oxidized fish oil plus ISF ( $P \leq 0.05$ ; Table 5). Piglets fed oxidized fish oil diet had higher concentrations of TNF- $\alpha$  in jejunal mucosa than piglets fed fresh fish oil ( $P < 0.05$ ), whereas its concentrations were numerically lower in piglets fed oxidized fish oil plus ISF diet, but did not differ from those fed fresh fish oil. All piglets fed oxidized fish oil diets had significantly lower IL-4 concentrations than those fed fresh fish oil, but no significant differences from the groups supplemented with oxidized fish oil and oxidized fish oil plus ISF. There were no significant differences in jejunal concentrations of IL-6, IL-8, IL-10, or IFN- $\gamma$  among the three treatments.

### 3.5 Jejunal mucosal NF- $\kappa$ B, iNOS, NO, and Caspase-3

Piglets fed oxidized fish oil diet had higher jejunal concentrations of NF- $\kappa$ B, iNOS and Caspase-3, and lower NO than piglets fed fresh fish oil ( $P \leq 0.05$ ; Table 6), and supplemental ISF decreased the concentrations of NF- $\kappa$ B in jejunal mucosa and partially

offset these differences on the concentrations of iNOS, Caspase-3 and NO between the two groups fed fresh fish oil and oxidized fish oil.

## 4 Discussion

This is the first study to demonstrate that dietary ISF could partly alleviate the oxidative stress induced by oxidized fish oil by improving the intestinal morphology, antioxidant capacity, and immune function in young piglets. Our findings support the potential of ISF as an intestinal protective agent in young piglets with its attenuation of intestinal damage induced by oxidative stress. Although several studies have evaluated the effectiveness of ISF on piglets (de Wilde *et al.*, 2004; Mau *et al.*, 2008; Goerke *et al.*, 2012), none of these studies examined the effectiveness of ISF treatment on the oxidative stress and immune function of the piglets.

Numerous studies have shown that feeding oxidized fish oil to experimental animals can result in excessive production of ROS and MDA, the reduction of SOD, and the induction of oxidative stress (John *et al.*, 1959; Liu and Lee, 1998; Aw, 1999; VázquezAñón and Jenkins, 2007). In the present

**Table 5 Effect of dietary soybean isoflavone addition on the cell-mediated immunologic factors of jejunal mucosa<sup>1</sup> in young piglets fed a diet with oxidized fish oil**

Treatment <sup>2</sup>	sIgA ( $\mu$ g/ml)	IL-1 $\beta$ (pg/ml)	TNF- $\alpha$ (pg/ml)	IL-2 (pg/ml)	IL-4 (ng/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	IL-10 (ng/ml)	IFN- $\gamma$ (ng/ml)
FFO	81.59 <sup>a</sup>	108.90 <sup>b</sup>	30.55 <sup>b</sup>	112.86 <sup>b</sup>	50.67 <sup>a</sup>	27.69	134.84	4.63	6.22
OFO	50.06 <sup>b</sup>	149.70 <sup>a</sup>	44.42 <sup>a</sup>	136.09 <sup>a</sup>	26.43 <sup>c</sup>	29.54	137.61	5.99	6.76
OFO+20 ISF	78.90 <sup>a</sup>	111.23 <sup>b</sup>	42.68 <sup>ab</sup>	108.25 <sup>b</sup>	35.88 <sup>b</sup>	30.81	106.13	4.92	7.96
SEM	9.41	9.55	9.55	7.09	3.76	4.23	11.60	0.71	0.81
<i>P</i> -value	0.054	0.030	0.042	0.041	0.002	0.872	0.139	0.386	0.350

<sup>a,b,c</sup> Means ( $n=6$ ) in a column with different superscripts are significantly different ( $P \leq 0.05$ ). <sup>1</sup> Data are mean concentrations in supernatants of 1:10 homogenates. <sup>2</sup> Diets are designated as FFO for fresh fish oil diet or OFO for oxidized fish oil diet; 20 ISF is supplemented with 20 mg/kg soybean isoflavone. sIgA: secretory immunoglobulin A; IL: interleukin; TNF- $\alpha$ : tumor necrosis factor  $\alpha$ ; IFN- $\gamma$ : interferon  $\gamma$ ; SEM: standard error of the mean

**Table 6 Effect of dietary soybean isoflavone addition on the co-oxidation and immune-related factors of jejunal mucosa<sup>1</sup> in young piglets fed a diet with oxidized fish oil**

Treatment <sup>2</sup>	NO ( $\mu$ g/ml)	NF- $\kappa$ B (pg/ml)	iNOS (ng/ml)	Caspase-3 ( $\mu$ g/ml)
FFO	8.67 <sup>a</sup>	66.06 <sup>b</sup>	12.11 <sup>b</sup>	67.77 <sup>b</sup>
OFO	5.51 <sup>b</sup>	81.93 <sup>a</sup>	17.09 <sup>a</sup>	81.04 <sup>a</sup>
OFO+20 ISF	6.77 <sup>ab</sup>	73.61 <sup>b</sup>	16.03 <sup>a</sup>	72.34 <sup>ab</sup>
SEM	0.80	6.73	1.10	7.05
<i>P</i> -value	0.054	0.052	0.019	0.055

<sup>a,b</sup> Means ( $n=6$ ) in a row with different superscripts are significantly different ( $P \leq 0.05$ ). <sup>1</sup> Data are mean concentrations in supernatants of 1:10 homogenates. <sup>2</sup> Diets are designated as FFO for fresh fish oil diet or OFO for oxidized fish oil diet; 20 ISF is supplemented with 20 mg/kg soybean isoflavone. NO: nitric oxide; NF- $\kappa$ B: nuclear factor  $\kappa$  B; iNOS: inducible nitric oxide synthase; SEM: standard error of the mean

study, the content of MDA in the jejunal mucosa of piglets fed oxidized fish oil was significantly higher, and the activity of GSH-Px and ratio of GSH/GSSG were lower than those of piglets fed diets containing fresh fish oil. The lower intestinal morphometry, especially the reduction in duodenal villous height, is consistent with oxidative damage to the intestine, thus providing a potentially valuable model of the influence of oxidative stress on intestinal development and the associated function.

Previous studies have indicated that ISF improved the antioxidant activity in the breast muscle of male broilers fed oxidized fish oil (Jiang *et al.*, 2007). Liu *et al.* (2005) also used soy ISF extracts to elevate the activities of many antioxidant enzymes in rats fed diets containing partially oxidized oil and proved that ISF alleviated the negative effect of oxidized fish oil. Our present results show that the balance of antioxidant/oxidant status in the intestine of piglets is disturbed by feeding oxidized fish oil and partially reverted by feeding ISF. The concentrations of MDA and GSSG in piglets fed oxidized fish oil increased, while they were decreased by the inclusion of ISF in the diet. The activity of GSH-Px changed in the opposite direction. These findings indicate that intestinal oxidative stress provoked by feeding oxidized fish oil is reduced by ISF.

Our present results show that the final BW and ADG are significantly lower in piglets fed oxidized fish oil diet, but the addition of ISF restores BW and ADG to the levels of the control group fed fresh fish oil. Note that the high values of the G:F ratio result from the feed being of very high dry matter (DM) content compared to the much lower expected DM content of these young pigs. As reported earlier with broilers (Jiang *et al.*, 2007) and piglets (Yuan, 2007), the addition of ISF offsets the deleterious effect of oxidized fish oil on growth performance.

Growth retardation of weaned piglets was related to the reduction of villous height (Cera *et al.*, 1988). Yuan (2007) found that oxidative stress caused by feeding oxidized fish oil decreased the villous dimensions in piglets and their growth. The present study demonstrates that the addition of oxidized fish oil to piglets decreases the duodenal villous height, and 20 mg/kg ISF protects the duodenal villi from the deleterious effects of oxidized fish oil. Presumably, ISF, by stimulating antioxidant function, confers

some degree of protection on the intestinal mucosa, helping to improve the growth performance.

It has been reported that atrophic changes in the intestinal mucosa, like those caused here by oxidized fish oil, provoke immediate or delayed-type hypersensitivity and mucosal inflammation (Li *et al.*, 1990; 1991). Our results show that sIgA is decreased by feeding oxidized fish oil, whereas supplemental ISF markedly increases the intestinal IgA levels, probably by augmenting B- and T-cell counts (Fan *et al.*, 2010). The IgA-mediated intestinal immune response is controlled by the balance between opposing actions of the Th2-type IgA-stimulating and the Th1-type IgA-inhibiting cytokines from their respective lymphocyte subsets (Kudsk, 2001; Pruett, 2003; Shang *et al.*, 2004). Th1-mediated cytokines such as IL-1 $\beta$ , IL-2, and TNF- $\alpha$  enhance cell-mediated immunity, and are produced by Th1 lymphocytes. A predominant Th1 effect results in the activation of T lymphocytes. Th2 cytokines such as IL-4 and IL-10 enhance humoral immunity, and are produced by Th2 lymphocytes (Kalinski and Moser, 2005). A predominant effect of Th2 lymphocytes results in the activation of B lymphocytes and upregulation of antibody production. There was a shift from Th1 to Th2 profiles of cytokines in the jejunal extracts from the ISF-fed piglets, as the levels of IL-4 declined significantly and those of IL-1 $\beta$ , IL-2, and TNF- $\alpha$  increased, compared to piglets fed oxidized fish oil alone. The pattern of changes in IL-4 levels closely approximated those of IgA (Fan *et al.*, 2010). Deng *et al.* (2010) reported that diets supplemented with antioxidants decreased the serum levels of inflammatory cytokines in post-weaning piglets. Morimoto *et al.* (2009) found less inflammation and colonic tissue damage in mice supplemented with ISF than in control mice with experimental colitis, and Kim *et al.* (2004) found that ISF inhibited IL-1-induced upregulation of NF- $\kappa$ B activation.

Pathogens that cause acute inflammation activate the NF- $\kappa$ B and iNOS pathways, thereby regulating genes encoding pro-inflammatory cytokines, chemokines, and adhesion molecules (Jelínková *et al.*, 2004). Gookin *et al.* (2002) demonstrated that the villous epithelium is the predominant site of iNOS expression and that the lack of iNOS in porcine decreases the inflammatory responses (Pampusch *et al.*, 1998). Epithelial iNOS expression has likewise been reported

in mice experimentally infected with *Cryptosporidium parvum* (Leitch and He, 1999). Other reports also showed that the epithelium is capable of rapid synthesis of iNOS after acute gut injury (Banan *et al.*, 2001; Gookin *et al.*, 2002). The novelty of the present study is that the concentrations of iNOS and Caspase-3 were increased by oxidized fish oil but the increase was partially alleviated by supplementation of ISF. Caspase-3 also is activated by oxidative stress and helps to increase the inflammatory response (Ozacak *et al.*, 2014), which is consistent with our result. In addition, the concentration of NF- $\kappa$ B in this present study increased significantly in the young piglets fed oxidized fish oil diets, consistent with the hypothesis that oxidative stress in the intestinal mucosa increases ROS and then increases expression of pro-inflammatory cytokines via activating NF- $\kappa$ B (Aw, 1999). NF- $\kappa$ B was partially reduced in the piglets fed oxidized fish oil plus ISF compared to that in the piglets fed oxidized fish oil alone, a finding consistent with that from other models (Valachovicova *et al.*, 2004; Li *et al.*, 2005). The most striking benefit was the reduction in NF- $\kappa$ B seen in piglets fed oxidized fish oil plus ISF diets. It appears that NO modulates the phenotype of T-helper cells to promote a shift from Th1 to Th2, accompanied by a decline in pro-inflammatory mediators including IL-2 and by an increase in anti-inflammatory mediators such as IL-4 (Chang *et al.*, 1997; Taylor-Robinson, 1997; Daniel *et al.*, 2006). NO decreased in the jejunal mucosa of piglets fed oxidized fish oil alone, while the decrease was less in the piglets fed oxidized fish oil plus ISF. NO is also involved in activation of Caspase-3 (Parikh *et al.*, 2003), and in this present research NO was decreased in piglets fed oxidized fish oil; probably, the activation of Caspase-3 needs the consumption of NO.

The antioxidant properties of ISF are both direct, by scavenging or quenching oxygen free radicals thereby inhibiting MDA production, and indirect, by preventing increased membrane permeability resulting from oxidative stress, in many tissues including liver and plasma (Jiang *et al.*, 2007; 2011). In conclusion, our results demonstrated that addition of oxidized fish oil increased MDA and decreased GSH-Px activity in the jejunal mucosa, changed the cytokines in jejunal extracts from a pro- to an anti-inflammatory profile, and increased sIgA and de-

creased NF- $\kappa$ B concentrations. Collectively, these data show that dietary inclusion of moderate amounts (20 mg/kg) of ISF is an effective way of reducing oxidative stress in the small intestine of young piglets fed diets containing oxidized fish oil.

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### Compliance with ethics guidelines

Lin HUANG, Xian-yong MA, Zong-yong JIANG, You-jun HU, Chun-tian ZHENG, Xue-fen YANG, Li WANG, and Kai-guo GAO declare that they have no conflict of interest.

This study was conducted according to the Chinese Guidelines for the Use of Experimental Animals and Animal Welfare (Science and Technology Ministry of China, 2006) and approved by Animal Experimental Committee of Institute of Animal Science, Guangdong Academy of Agricultural Sciences. All institutional and national guidelines for the care and use of laboratory animals were followed.

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## 中文概要

**题目:** 大豆异黄酮对饲喂氧化鱼油新生仔猪肠道抗氧化功能及细胞因子的影响

**目的:** 研究大豆异黄酮对氧化鱼油造成仔猪氧化应激的缓解作用, 并初步探讨其作用机制。

**创新点:** 首次在仔猪肠道氧化应激模型中证明大豆异黄酮可缓解仔猪氧化应激, 且此作用与提高其免疫功能相关。

**方法:** 将 72 头平均体重约 1.8 kg 的 4 日龄杜×(长×大)三元杂公猪随机分为 3 组, 每组重复 6 次, 每次重复包括 4 头猪: 对照组(饲喂 50 g/kg 新鲜鱼油), 处理组 1(添加 50 g/kg 氧化鱼油)和处理组 2(添加 50 g/kg 氧化鱼油+20 mg/kg 大豆异黄酮); 基

础日粮根据 NRC (2012)《猪的营养需要量》配制, 每 3 小时饲喂一次, 记录每天采食量; 实验期 21 天结束后, 称重, 计算平均日采食量、平均日增重及料重比。用苏木精-伊红染色法观察小肠形态结构, 并计算绒毛高度和隐窝深度, 应用南京建成试剂盒测定肠道中抗氧化酶的活性, 应用酶联免疫吸附测定 (ELISA) 试剂盒测定细胞炎症相关因子和细胞凋亡相关因子的表达。

**结论:** 饲喂氧化鱼油引起新生仔猪肠道产生氧化应激, 引起仔猪生长性能下降、肠道发育受损、细胞凋亡增加及免疫功能失调。而添加大豆异黄酮能减缓氧化鱼油造成的肠道损伤, 提高肠道粘膜完整性, 提高肠道粘膜细胞抗氧化功能和免疫功能。

**关键词:** 仔猪; 氧化鱼油; 生长性能; 大豆异黄酮; 抗氧化功能