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Effect of flaxseed oil on muscle protein loss and carbohydrate oxidation impairment in a pig model after lipopolysaccharide challenge

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Abstract

Flaxseed oil is rich in α-linolenic acid (ALA), which is the metabolic precursor of EPA and DHA. The present study investigated the effect of flaxseed oil supplementation on lipopolysaccharide (LPS)-induced muscle atrophy and carbohydrate oxidation impairment in a piglet model. Twenty-four weaned pigs were used in a 2 × 2 factorial experiment including dietary treatment (5 % maize oil *v.* 5 % flaxseed oil) and LPS challenge (saline *v.* LPS). On day 21 of treatment, the pigs were injected intraperitoneally with 100 μg/kg body weight LPS or sterile saline. At 4 h after injection, blood, gastrocnemius muscle and longissimus dorsi muscle were collected. Flaxseed oil supplementation increased ALA, EPA, total *n*-3 PUFA contents, protein:DNA ratio and pyruvate dehydrogenase complex quantity in muscles ($P < 0{\cdot}05$). In addition, flaxseed oil reduced mRNA expression of toll-like receptor (TLR) 4 and nucleotide-binding oligomerisation domain protein (NOD) 2 and their downstream signalling molecules in muscles and decreased plasma concentrations of TNF-*α*, IL-6 and IL-8, and mRNA expression of TNF-*α*, IL-1*β* and IL-6 ($P < 0{\cdot}05$). Moreover, flaxseed oil inclusion increased the ratios of phosphorylated protein kinase B (Akt) 1:total Akt1 and phosphorylated Forkhead box O (FOXO) 1:total FOXO1 and reduced mRNA expression of FOXO1, muscle RING finger (MuRF) 1 and pyruvate dehydrogenase kinase 4 in muscles ($P < 0{\cdot}05$). These results suggest that flaxseed oil might have a positive effect on alleviating muscle protein loss and carbohydrates oxidation impairment induced by LPS challenge through regulation of the TLR4/NOD and Akt/FOXO signalling pathways.

Key words: Protein kinase B/Forkhead box O signalling pathways: Carbohydrate oxidation impairment: Flaxseed oil: Muscle atrophy: Toll-like receptor 4/nucleotide-binding oligomerisation domain protein signalling pathway

Skeletal muscle is the most widely distributed tissue in the body, which is involved in many important biological functions. It is also an important site to regulate the whole-body metabolism. However, pro-inflammatory cytokines such as TNF-*α*, IL-1*β* and IL-6 may cause muscle atrophy and metabolic disturbance, which is one of the prominent features during inflammation and infection and characterised by muscle strength loss, protein degradation and carbohydrate metabolism impairment[1].

During inflammation and infection, pattern recognition receptors such as toll-like receptors (TLR) and nucleotide-binding oligomerisation domain proteins (NOD), as critical components of innate immune response, are activated to produce pro-inflammatory cytokines[2,3]. These pro-inflammatory cytokines directly induce muscle protein degradation or lead to muscle atrophy through inhibiting muscle protein kinase B (Akt) and activating Forkhead box O (FOXO) transcription factors and further activating FOXO target genes such as E3-ubiquitin ligases muscle atrophy F-box (MAFbx) and muscle RING finger (MuRF) 1, which is the main pathway of protein degradation in skeletal muscle[1]. In addition, the activated FOXO impairs carbohydrate oxidation by activating pyruvate dehydrogenase kinase (PDK) 4 and then inhibiting pyruvate dehydrogenase complex (PDC) activity[4].

The inflammatory response (inflammation) is part of innate immunity. It occurs when tissues are injured by bacteria, trauma, toxins, heat or any other cause[5]. Long-chain *n*-3 PUFA such as DHA (22 : 6*n*-3) and EPA (20 : 5*n*-3) rich in deep-sea fish oil can inhibit inflammatory response and exert beneficial effects on human clinical studies and animal experiments[6,7]. In addition,

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Abbreviations: Akt, protein kinase B; ALA, α-linolenic acid; BUN, blood urea N; CCL2, CC chemokine ligand 2; CD14, cluster of differentiation factor 14; FOXO, Forkhead box O; LBP, lipopolysaccharide-binding protein; LD, longissimus dorsi; LPS, lipopolysaccharide; MAFbx, muscle atrophy F-box; MuRF, muscle RING finger; NOD, nucleotide-binding oligomerisation domain protein; p-Akt, phosphorylated Akt; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; p-FOXO, phosphorylated FOXO; RIPK, receptor-interacting serine/threonine-protein kinase; t-FOXO, total FOXO; TLR, toll-like receptor; TRAF, TNF-α receptor-associated factor.

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n-3 PUFA (EPA or DHA) alleviates muscle atrophy[8,9], improves muscle protein synthesis in older, healthy young and middle-aged adults[10,11] and decreases PDK activity in human skeletal muscle[12]. So, the general public is recommended to consume fish oil in many nutritional guidelines[13]. However, the use of fish oil on clinic is often limited because of the characteristics of easy oxidation[14]. So, alternative sources of *n*-3 PUFA existing in plants receive attention. Flaxseed oil, a traditional edible oil, is rich in α-linolenic acid (ALA; 18 : 3*n*-3). ALA is the metabolic precursor of EPA and DHA[15]. Recently, emerging literatures have shown that flaxseed oil inclusion in the diet decreased inflammatory response and exerted anti-inflammatory effects[16,17]. However, unlike fish oil, few studies have been conducted to explore the effect of flaxseed oil on muscle atrophy and carbohydrate oxidation impairment.

The weaned piglet model is suitable for human nutrition research, particularly child and adolescent with muscles undergoing rapid growth[18,19]. Lipopolysaccharide (LPS) is often utilised to establish the model of endotoxaemia[1]. Therefore, in the present study, our objective was to investigate whether flaxseed oil had a protective effect on muscle atrophy and carbohydrates oxidation impairment caused by LPS challenge and to elucidate its molecular mechanism(s) in the weaned piglet model.

Methods

Animal and experimental design

The Animal Care and Use Committee of Wuhan Polytechnic University approved the animal use protocol for this research. Twenty-four weaned crossbred castrated barrows (Duroc × Large White × Landrace; 8·91 (SEM 0·21) kg; 35 (SEM 1) d of age) were randomly assigned to four treatment groups and allowed *ad libitum* access to water and feed during a 21-d experimental study; each treatment group had six replicated pens, and each pen had one pig, which was based on our previous studies[7,20]. In the whole experimental period, all pigs were placed individually in pens (1·80 × 1·10 m) and in good health condition. The ambient temperature was maintained at 22–25°C, and the living environment was in accordance with animal welfare guidelines.

The experiment was conducted as a 2 × 2 factorial arrangement of treatments including dietary treatment (5 % maize oil (Xiwang Food Company) *v*. 5 % flaxseed oil (Yulongxiang Grain and Oil Company)) and LPS (*Escherichia coli* serotype 055:B5, Sigma Chemical) challenge (saline *v*. LPS). On day 21, half of the pigs in each dietary treatment were injected intraperitoneally with 100 μg/kg body weight LPS or the same volume of 0·9 % sterile saline solution according to our previous study[7]. This dose of LPS could cause acute tissue injury in weaned pigs. The ingredient composition of the basal diet and the fatty acid composition of maize oil and flaxseed oil were shown in our previous study[20].

Sample collection

At 4 h after LPS or saline injection, blood samples were harvested into 10-ml heparinised vacuum tubes (Becton Dickinson Vacutainer System) and centrifuged (3500 ***g*** for 10 min) to collect plasma. Plasma from each pig was stored at –80°C until further analysis. After blood collection, pigs were slaughtered under anaesthesia with an intravenous injection of pentobarbital sodium (80 mg/kg body weight), and a portion of gastrocnemius muscle and longissimus dorsi (LD) muscle (approximately 20 g, respectively) was removed and frozen in liquid N_2 immediately and then stored at −80°C for mRNA and protein expression analysis. Ooi *et al.* found piglets with muscle fibre atrophy had high levels of MAFbx in both gastrocnemius muscle and LD muscle[21]. LPS can induce muscle atrophy by up-regulating mRNA expression of MAFbx and MuRF1[22–24]. Therefore, these muscles were selected to study muscle atrophy[21,25]. In addition, the time point of 4 h after LPS or saline injection was selected according to published papers[26–28].

Fatty acids composition in muscles

Total fat was prepared from muscles according to GB/T 14772-2008[29]. The composition of fatty acids in muscles was analysed according to the method of Sun *et al.*[30]. Briefly, the muscle (about 5 g) was extracted in a Soxhlet extractor with diethyl ether for 6–12 h; then, the extraction was concentrated in water bath at 70–80°C and dried at 103°C for 1 h. Undecanoic acid (C11 : 0, 1 ml, served as the internal standard) and acetyl chloride methanol solution (4 ml) were added to the extracted fat and then placed in an 80°C water for 2 h. After cooling, 5 ml potassium carbonate solution (7 %) was added into the mix and then mixed and centrifuged at 1200 ***g*** for 5 min (4°C). The resulting supernatant was put into a DB-23 capillary column (60·0 μm × 250 μm×0·25 μm; Agilent Technoligies) to analyse the fatty acid composition by gas chromatograph (Agilent 6890; Agilent Technoligies).

Glucose, blood urea nitrogen, insulin, cortisol, glucagon, TNF-α, IL-6 and IL-8 concentrations in plasma

Plasma glucose level was determined by the glucose GOD-PAP assay kit (DiaSys Diagnostic Systems GmbH). Plasma blood urea N (BUN) concentrations were measured by a commercial urea assay kit (Nanjing Jiancheng Bioengineering Institute). Plasma insulin, cortisol and glucagon contents were measured using commercially available ^{125}I RIA assay kits (Beijing North Institute of Biological Technology). Plasma TNF-*α*, IL-6 and IL-8 levels were determined by using a commercially available porcine ELISA assay kit (R&D Systems). All experimental procedures were conducted according to the manufacturer's recommendations.

Protein and DNA contents in muscles

The muscle samples were homogenised with a tissue homogeniser (PT-3100D; Kinematica) in ice-cold PBS EDTA (0·05 M-Na_3PO_4, 2·0 M-NaCl, 2×10^{-3} M-EDTA, pH 7·4) using a 1:10 (w/v) ratio. Protein concentration of muscle homogenates was determined by a published method[31] using a detergent-compatible protein assay (Bio-Rad Laboratories) and bovine serum albumin as standards. Muscle DNA content was evaluated by a fluorometric assay[32].

Table 1. Primer sequences used for real-time PCR

Gene	Forward (5′–3′)	Reverse (5′–3′)	Product length (bp)	Accession numbers
TLR4	TCAGTTCTCACCTTCCTCCTG	GTTCATTCCTCACCCAGTCTTC	166	GQ503242.1
LBP	GAACACAGCCGAATGGTCTAC	GGAAGGAGTTGGTGGTCAGT	151	NM 001128435.1
CD14	CGTTTGTGGAGCCTGGAAG	TGCGGATGCGTGAAGTTG	226	NM_001097445.2
MyD88	GATGGTAGCGGTTGTCTCTGAT	GATGCTGGGGAACTCTTTCTTC	148	AB292176.1
IRAK1	CAAGGCAGGTCAGGTTTCGT	TTCGTGGGGCGTGTAGTGT	115	XM_003135490.1
TRAF6	CAAGAGAATACCCAGTCGCACA	ATCCGAGACAAAGGGGAAGAA	122	NM_001105286.1
NOD1	CTGTCGTCAACACCGATCCA	CCAGTTGGTGACGCAGCTT	57	AB187219.1
NOD2	GAGCGCATCCTCTTAACTTTCG	ACGCTCGTGATCCGTGAAC	66	AB195466.1
RIPK2	CAGTGTCCAGTAAATCGCAGTTG	CAGGCTTCCGTCATCTGGTT	206	XM_003355027.1
NF-κB	AGTACCCTGAGGCTATAACTCGC	TCCGCAATGGAGGAGAAGTC	133	EU399817.1
TNF-α	TCCAATGGCAGAGTGGGTATG	AGCTGGTTGTCTTTCAGCTTCAC	67	NM_214022.1
IL-1β	GCTAACTACGGTGACAACAATAATG	CTTCTCCACTGCCACGATGA	186	NM_214055.1
IL-6	AAGGTGATGCCACCTCAGAC	TCTGCCAGTACCTCCTTGCT	151	JQ839263.1
IL-8	ACAGCAGTAACAACAACAAG	GACCAGCACAGGAATGAG	117	NM_213867.1
IL-10	AAGTCGCTTACACTCATAGGAA	CCAGAGGAATTGAATTGCTTCT	197	NM_214041.1
CCL2	TTGAATCCTCATCCTCCAGCAT	GTAAGTGTATAGCAGCAGGTGAC	136	NM_214214.1
CXCL16	CTCCGACCGATCACGACAT	CTGCTGCCTCCACATACACT	112	NM_213811.1
COX2	ATGATCTACCCGCCTCACAC	AAAAGCAGCTCTGGGTCAAA	284	AY028583
Akt1	GAAGAAGGAGGTCATCGT	GGACAGGTGGAAGAAGAG	178	NM_001159776.1
FOXO1	TTCACCAGGCACCATCAT	GAGGAGAGTCGGAAGTAAGT	236	NM_214014.2
FOXO4	TGGAGTGTGACATGGATAAC	CTCATCTCTGAAGCAAGGAA	122	XM_003135172.3
MAFbx	TCACAGCTCACATCCCTGAG	GACTTGCCGACTCTCTGGAC	167	NM_001044588.1
MuRF1	ATGGAGAACCTGGAGAAGCA	ACGGTCCATGATCACCTCAT	219	FJ905227.1
PDK4	GCCTCAGTGGCATAAAAACC	CAGTGGTGGTAATACAAAGG	211	NM_001159306
GAPDH	CGTCCCTGAGACACGATGGT	GCCTTGACTGTGCCGTGGAAT	194	AF017079.1

TLR, toll-like receptor; *LBP*, lipopolysaccharide-binding protein; *CD14*, cluster of differentiation factor 14; *MyD88*, myeloid differentiation factor 88; *IRAK1*, IL-1 receptor-associated kinase 1; *TRAF6*, TNF receptor-associated factor 6; *NOD*, nucleotide-binding oligomerisation domain protein; *RIPK2*, receptor-interacting serine/threonine-protein kinase 2; *CCL2*, CC chemokine ligand 2; *COX2*, mitogen-inducible cyclo-oxygenase 2; *Akt1*, protein kinase B1; *FOXO*, Forkhead box O; *MAFbx*, muscle atrophy F-box; *MuRF1*, muscle RING finger 1; *PDK4*, pyruvate dehydrogenase kinase 4; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

Glycogen and lactate contents and pyruvate dehydrogenase complex quantity in muscles

Glycogen and lactate contents in muscles were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute). PDC quantity was analysed using a commercial ELISA kit (Yuanye Biotech Co., Ltd). All experimental procedures were conducted according to the manufacturer's recommendations.

mRNA abundance analysis by real-time PCR

The genes expression were measured as previously described[20]. Briefly, total RNA was isolated by the Trizol reagent (no. 9108, TaKaRa Biotechnology (Dalian) Co., Ltd). RNA quality for each sample was detected by agarose gel electrophoresis, and its concentration was determined by spectrophotometry based on the OD260:OD280 ratio. cDNA was synthesised using PrimeScript® RT reagent kit (no. RR047A, TaKaRa Biotechnology (Dalian) Co., Ltd) according to the manufacture's instruction. Real-time PCR assay for the target genes was carried out on a ABI 7500 Real-Time PCR System (Applied Biosystems, Life Technologies) using a SYBR® Premix Ex TaqTM (Tli RNaseH Plus) quantitative PCR kit (no. RR420A, TaKaRa Biotechnology (Dalian) Co., Ltd). The PCR cycling conditions were 95°C × 30 s, followed by forty cycles of 95°C × 5 s and 60°C × 34 s. The forward and reverse primers for the target genes were designed with Primer Premier 6.0 and synthesised by TaKaRa Biotechnology (Table 1), and the mRNA expression relative to a housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase; *GAPDH*) was calculated according to the $2^{-\triangle\triangle CT}$ method[33].

Protein abundance analysis by Western blot

Quantification of protein expression in muscles was performed as previously described[7,26]. Briefly, supernatant proteins were separated on 12 % SDS-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes for immunoblotting. The primary antibodies including total Akt (no. 9272), phosphorylated Akt (p-Akt, Ser473, no. 9271), total FOXO1 (t-FOXO1, no. 9454) and phosphorylated FOXO1 (p-FOXO1, Ser256, no. 9461) and the secondary antibodies had been described in Kang *et al.*[34]. In the present study, the same target protein, including total and phosphorylated proteins, was measured on the same gel by using their respective antibodies. The bands were analysed by densitometry using GeneTools software (Syngene), and the abundance of the phosphorylated proteins was normalised to the total protein contents.

Statistical analysis

The experimental data were analysed using the general linear model procedure of Statistical Analysis System appropriate for a 2 × 2 factorial design. The statistical model included the effects of diet (maize oil *v.* flaxseed oil) and LPS challenge (saline *v.* LPS) and their interactions. If there was a significant or a trend interaction observed between LPS challenge and diet, *post hoc* testing was conducted using Duncan's multiple comparison tests. Data were expressed as means and standard deviations. The statistical significance level for all analyses was set at $P \leq 0{\cdot}05$, and $0{\cdot}05 < P < 0{\cdot}10$ was considered as trends. A two-way multivariate ANOVA was used to determine the effect size (partial η^2) and the statistical power of the model. There was a statistically significant

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Table 2. Effects of flaxseed oil or maize oil supplementation on muscle fatty acid composition after 4 h *Escherichia coli* lipopolysaccharide (LPS) challenge in weaned piglets*
(Mean values and standard deviations, *n* 6 (one pig per pen))

	Saline				LPS						
	Maize oil		Flaxseed oil		Maize oil		Flaxseed oil		*P*		
Item	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Diet	LPS	Interaction
Gastrocnemius muscle											
14 : 0	1·14	0·12	1·00	0·06	1·08	0·10	1·02	0·06	0·010	0·539	0·234
16 : 0	22·38	1·12	20·42	0·56	22·42	0·65	20·63	0·52	<0·001	0·680	0·793
16 : 1*n*-7	2·13	0·37	2·02	0·37	2·10	0·12	1·89	0·38	0·247	0·582	0·704
18 : 0	11·76	0·80	12·89	0·74	11·85	0·55	12·99	1·07	0·003	0·781	0·996
cis 18 : 1*n*-9	28·03	0·84	25·18	2·22	27·93	1·86	24·99	1·97	0·001	0·846	0·949
cis 18 : 2*n*-6	27·54[a]	0·64	19·61[b]	1·11	26·09[c]	1·59	20·07[b]	1·25	<0·001	0·324	0·064
cis 18 : 3*n*-3	0·44	0·03	9·28	0·58	0·44	0·02	9·34	1·01	<0·001	0·912	0·901
20 : 4*n*-6	3·22	0·69	2·37	0·40	3·23	0·49	2·48	0·42	0·001	0·775	0·811
20 : 3*n*-3	0·03	0·05	0·91	0·04	0·02	0·03	0·95	0·12	<0·001	0·625	0·377
20 : 5*n*-3	0·16	0·04	1·16	0·24	0·16	0·04	1·25	0·18	<0·001	0·500	0·467
24 : 0	0·77[a]	0·41	1·70[c]	0·39	1·05[a,b]	0·12	1·35[b,c]	0·28	<0·001	0·783	0·024
22 : 6*n*-3	0·75	0·14	0·67	0·13	0·77	0·17	0·71	0·09	0·211	0·621	0·893
Total *n*-6 PUFA	31·10	1·26	22·22	1·49	29·63	2·01	22·83	1·69	<0·001	0·526	0·134
Total *n*-3 PUFA	1·39	0·15	12·03	0·83	1·39	0·21	12·26	1·14	<0·001	0·704	0·700
n-6:*n*-3	22·56	1·68	1·85	0·08	21·74	3·33	1·88	0·22	<0·001	0·609	0·586
Longissimus dorsi muscle											
14 : 0	1·15	0·19	1·13	0·07	1·10	0·14	1·06	0·09	0·632	0·293	0·851
16 : 0	23·42	1·38	21·90	0·61	23·60	0·78	21·96	0·47	<0·001	0·758	0·869
16 : 1*n*-7	2·15	0·57	1·91	0·38	1·87	0·10	1·80	0·46	0·375	0·277	0·612
18 : 0	12·65	1·13	14·19	0·95	13·39	0·41	14·40	1·16	0·005	0·252	0·506
cis 18 : 1*n*-9	27·24	3·61	25·55	2·70	25·50	2·06	24·37	2·94	0·254	0·241	0·818
cis 18 : 2*n*-6	25·70	2·81	19·43	0·53	25·86	1·97	19·45	1·49	<0·001	0·916	0·932
cis 18 : 3*n*-3	0·36	0·04	8·30	0·62	0·34	0·03	8·34	0·60	<0·001	0·979	0·861
20 : 4*n*-6	3·19	1·28	2·16	0·55	3·97	0·56	2·20	0·36	<0·001	0·232	0·280
20 : 3*n*-3	0·00[a]	0·00	0·86[b]	0·05	0·00[a]	0·00	0·79[c]	0·10	<0·001	0·087	0·087
20 : 5*n*-3	0·14	0·06	1·24	0·36	0·16	0·04	1·36	0·23	<0·001	0·482	0·612
24 : 0	0·40	0·12	0·90	0·23	0·50	0·21	1·04	0·19	<0·001	0·143	0·730
22 : 6*n*-3	0·60	0·21	0·50	0·17	0·79	0·15	0·51	0·03	0·011	0·154	0·191
Total *n*-6 PUFA	29·26	4·08	21·89	1·03	30·22	2·47	21·96	1·89	<0·001	0·648	0·692
Total *n*-3 PUFA	1·11	0·24	10·91	0·66	1·29	0·16	10·99	0·60	<0·001	0·503	0·796
n-6:*n*-3	26·74	2·27	2·01	0·14	23·57	2·29	2·00	0·14	<0·001	0·033	0·034

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
* Total *n*-6 PUFA and total *n*-3 PUFA corresponded to the sum of all the detected *n*-6 or *n*-3 PUFA.

interaction effect between diet and LPS on the combined dependent variables ($F = 3·287$, $P = 0·036$, Wilks' $\Lambda = 0·217$, partial $\eta^2 = 0·783$, the power to detect the effect was 0·789).

Results

Fatty acid composition in muscles

During the whole experimental period, there were no adverse events. The fatty acid composition in muscles is shown in Table 2. Flaxseed oil supplementation increased ALA, eicosatrienoic acid (C20 : 3*n*-3), EPA and total *n*-3 PUFA proportions in both gastrocnemius muscle and LD muscle ($P < 0·05$). However, its inclusion decreased linoleic acid (C18 : 2*n*-6), eicosatrienoic acid (C20 : 3*n*-6), arachidonic acid (C20 : 4*n*-6), total *n*-6 PUFA proportion and *n*-6:*n*-3 ratio in both gastrocnemius muscle and LD muscle ($P < 0·05$).

Glucose, blood urea nitrogen, insulin, cortisol, glucagon and proinflammatory cytokine concentrations in plasma

As shown in Table 3, the piglets challenged with LPS had lower plasma glucose and insulin contents and higher plasma BUN, cortisol, glucagon, TNF-α, IL-6 and IL-8 contents compared with the piglets injected with saline ($P < 0·05$). There was an interaction observed between LPS challenge and diet for plasma cortisol, IL-6 and IL-8 contents ($P < 0·05$) and a trend for plasma TNF-α content ($P < 0·1$). Compared with maize oil, flaxseed oil reduced plasma cortisol, IL-6, IL-8 and TNF-α contents in piglets challenged by LPS ($P < 0·05$); however, flaxseed oil had no effects on these variables in saline-treated piglets. In addition, there was no interaction observed between LPS challenge and diet for plasma glucose, BUN, insulin and glucagon contents. Flaxseed oil decreased BUN and glucagon contents in both saline-treated and LPS-challenged piglets ($P < 0·05$).

Protein:DNA ratio in muscles

As shown in Table 4, LPS challenge decreased protein:DNA ratio in gastrocnemius muscle ($P < 0·05$). There was a trend for diet × LPS interaction for protein:DNA ratio in gastrocnemius muscle ($P < 0·1$). Compared with maize oil, flaxseed oil inclusion had no effect on this ratio in gastrocnemius muscle in piglets treated with saline, whereas its inclusion elevated this ratio in gastrocnemius muscle in piglets challenged by LPS ($P < 0·05$).

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Table 3. Effects of flaxseed oil or maize oil supplementation on plasma glucose, blood urea nitrogen (BUN), insulin, cortisol, glucagon, and proinflammatory cytokine concentrations after 4 h *Escherichia coli* lipopolysaccharide (LPS) challenge in weaned piglets
(Mean values and standard deviations, *n* 6 (one pig per pen))

	Saline				LPS						
	Maize oil		Flaxseed oil		Maize oil		Flaxseed oil		*P*		
Item	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Diet	LPS	Interaction
Glucose (mmol/l)	4·73	0·53	4·59	0·77	2·94	0·80	3·05	1·13	0·981	<0·001	0·717
BUN (mmol/l)	2·73	0·68	1·90	0·55	3·61	1·24	2·64	0·68	0·016	0·027	0·830
Insulin (μIU/ml)	15·13	4·59	13·22	3·29	11·41	3·27	9·93	1·55	0·231	0·019	0·875
Cortisol (ng/ml)	67·12[a]	25·20	69·67[a]	20·88	289·38[c]	14·95	201·85[b]	70·69	0·016	<0·001	0·012
Glucagon (pg/ml)	120·56	14·56	93·38	7·37	427·18	154·12	296·02	58·28	0·030	<0·001	0·140
TNF-α (pg/ml)	ND[a]		ND[a]		2903[c]	134·59	2397[b]	455·96	0·009	<0·001	0·056
IL-6 (pg/ml)	ND[a]		ND[a]		13266·15[c]	1869·42	9166·40[b]	3831·09	0·021	<0·001	0·043
IL-8 (pg/ml)	103·11[a]	20·15	120·75[a]	34·60	2317·39[c]	1196·90	1035·39[b]	550·29	0·029	<0·001	0·025

ND, not detectable.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different (P < 0·05).

Table 4. Effects of flaxseed oil or maize oil supplementation on protein/DNA after 4 h *Escherichia coli* lipopolysaccharide (LPS) challenge in weaned piglets
(Mean values and standard deviations, *n* 6 (one pig per pen))

	Saline				LPS						
	Maize oil		Flaxseed oil		Maize oil		Flaxseed oil		*P*		
Item	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Diet	LPS	Interaction
Gastrocnemius muscle protein/DNA (mg/μg)	0·434[b]	0·054	0·454[b]	0·047	0·327[a]	0·060	0·431[b]	0·061	0·013	0·009	0·082
Longissimus dorsi muscle protein/DNA (mg/μg)	0·456	0·105	0·412	0·020	0·465	0·091	0·476	0·067	0·609	0·257	0·397

[a,b] Mean values within a row with unlike superscript letters were significantly different (P < 0·05).

Table 5. Effects of flaxseed oil or maize oil supplementation on muscle metabolites after 4 h *Escherichia coli* lipopolysaccharide (LPS) challenge in weaned piglets
(Mean values and standard deviations, *n* 6 (one pig per pen))

	Saline				LPS						
	Maize oil		Flaxseed oil		Maize oil		Flaxseed oil		*P*		
Item	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Diet	LPS	Interaction
Gastrocnemius muscle											
Glycogen (mg/g tissue)	35·26[a]	3·58	97·92[c]	20·22	55·82[b]	5·11	39·68[a,b]	7·53	0·007	0·020	<0·001
Lactate (mmol/g protein)	9·42	2·67	16·21	3·56	14·52	6·46	27·60	7·31	0·009	0·020	0·308
PDC (IU/mg protein)	14·30[a]	2·57	33·36[b]	2·89	15·57[a]	3·74	44·56[c]	3·63	<0·001	0·006	0·045
Longissimus dorsi muscle											
Glycogen (mg/g tissue)	32·95[a,b]	4·94	39·58[b]	4·92	39·44[b]	4·92	32·14[a]	2·53	0·917	0·881	0·055
Lactate (mmol/g protein)	19·40[a]	2·51	38·41[b,c]	2·66	38·84[b]	5·43	45·68[c]	2·63	<0·001	<0·001	0·031
PDC (IU/mg protein)	15·48	4·28	16·90	5·14	27·00	11·55	25·32	3·80	0·621	0·078	0·886

PDC, pyruvate dehydrogenase complex.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different (P < 0·05).

Glycogen and lactate contents and pyruvate dehydrogenase complex quantity in muscles

As shown in Table 5, LPS challenge increased lactate content in both gastrocnemius muscle and LD muscle ($P < 0·05$) and PDC quantity in gastrocnemius muscle ($P < 0·05$) and led to an increasing trend in PDC quantity in LD muscle ($P < 0·1$). There was an interaction between LPS challenge and diet for glycogen content and PDC quantity in gastrocnemius muscle ($P < 0·05$) and lactate content in LD muscle ($P < 0·05$), and there was a trend for diet × LPS interaction for glycogen content in LD muscle ($P < 0·1$). Compared with maize oil, flaxseed oil increased glycogen content in gastrocnemius muscle in saline-treated piglets ($P < 0·05$). In addition, flaxseed oil increased lactate content in LD muscle and PDC quantity in gastrocnemius muscle in both saline-treated and LPS-challenged piglets ($P < 0·05$).

mRNA expression of key genes in toll-like receptor 4 and nucleotide-binding oligomerisation domain proteins signalling pathways in muscles

As shown in Table 6, LPS challenge increased the mRNA expression of TLR4, LPS-binding protein (LBP), cluster of differentiation factor 14 (CD14), myeloid differentiation factor 88, NOD2, receptor-interacting serine/threonine-protein kinase (RIPK) 2, NF -κB, IL-1β, IL-6, IL-8, IL-10, CC chemokine ligand 2 (CCL2),

Table 6. Effects of flaxseed oil or maize oil supplementation on muscle mRNA expression of toll-like receptor 4 (*TLR4*) and nucleotide-binding oligomerisation domain proteins (*NOD*) and their downstream signals after 4 h *Escherichia coli* lipopolysaccharide (LPS) challenge in weaned piglets*
(Mean values and standard deviations, *n* 6 (one pig per pen))

	Saline				LPS				*P*		
	Maize oil		Flaxseed oil		Maize oil		Flaxseed oil				
Item	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Diet	LPS	Interaction
Gastrocnemius muscle											
TLR4	1·00	0·49	0·91	0·29	1·54	0·56	1·47	0·28	0·653	0·005	0·928
LBP	1·00[a]	0·27	1·80[a]	0·55	17·48[c]	2·34	10·17[b]	2·55	<0·001	<0·001	<0·001
CD14	1·00[a]	0·38	0·66[a]	0·16	4·45[c]	0·57	2·06[b]	0·51	<0·001	<0·001	<0·001
MyD88	1·00	0·45	1·01	0·29	2·14	0·23	2·17	0·40	0·900	<0·001	0·936
IRAK1	1·00	0·14	0·88	0·19	1·02	0·16	1·02	0·17	0·393	0·249	0·344
TRAF6	1·00	0·25	0·85	0·19	1·35	0·19	1·00	0·19	0·007	0·007	0·262
NOD1	1·00	0·32	0·89	0·08	1·03	0·16	0·87	0·20	0·126	0·969	0·788
NOD2	1·00	0·41	0·70	0·09	1·99	0·72	1·45	0·21	0·027	<0·001	0·512
RIPK2	1·00	0·21	0·84	0·18	2·78	0·45	2·13	0·58	0·019	<0·001	0·137
NF-κB p65	1·00	0·18	0·89	0·21	1·33	0·22	1·28	0·25	0·408	0·001	0·715
TNF-α	1·00	0·63	0·95	0·15	1·24	0·80	1·25	0·48	0·924	0·265	0·907
IL-1β	1·00[a]	0·71	1·14[a]	0·40	26·48[c]	9·66	12·12[b]	6·09	0·006	<0·001	0·006
IL-6	1·00[a]	0·37	0·74[a]	0·14	63·24[c]	23·06	30·98[b]	18·95	0·015	<0·001	0·016
IL-8	1·00	0·44	2·06	0·88	11·17	6·99	10·78	1·36	0·823	<0·001	0·624
IL-10	1·00	0·34	4·16	0·37	6·00	0·39	10·11	2·11	<0·001	<0·001	0·351
CCL2	1·00[a]	0·35	1·77[a]	0·21	9·17[b]	2·36	12·48[c]	0·52	<0·001	<0·001	0·020
CXCL16	1·00	0·32	0·98	0·20	2·95	0·48	2·98	0·87	0·985	<0·001	0·900
COX2	1·00[a]	0·39	1·12[a]	0·32	5·07[c]	1·79	2·86[b]	1·47	0·044	<0·001	0·026
Longissimus dorsi muscle											
TLR4	1·00[a]	0·29	1·03[a]	0·33	1·74[c]	0·45	1·07[b]	0·22	0·031	0·010	0·018
LBP	1·00[a]	0·23	1·96[b]	0·25	2·68[c]	0·84	1·39[a,b]	0·35	0·718	0·053	<0·001
CD14	1·00[a]	0·32	0·88[a]	0·19	3·59[c]	0·64	1·68[b]	0·11	<0·001	<0·001	<0·001
MyD88	1·00	0·44	0·88	0·17	2·45	0·41	1·87	0·33	0·024	<0·001	0·126
IRAK1	1·00	0·24	0·75	0·09	0·97	0·17	0·94	0·11	0·003	0·165	0·353
TRAF6	1·00	0·19	0·81	0·19	1·18	0·15	0·85	0·19	0·048	0·259	0·112
NOD1	1·00[a,b]	0·22	1·41[b]	0·68	1·14[a,b]	0·29	0·82[a]	0·12	0·808	0·175	0·033
NOD2	1·00[a]	0·53	1·00[a]	0·37	2·98[c]	0·71	1·75[b]	0·54	0·013	<0·001	0·012
RIPK2	1·00	0·22	0·75	0·23	1·97	0·40	1·62	0·20	0·014	<0·001	0·674
NF-κB p65	1·00	0·20	0·92	0·10	1·41	0·23	1·20	0·10	0·046	<0·001	0·360
TNF-α	1·00	0·41	0·79	0·16	1·41	0·51	0·76	0·46	0·018	0·275	0·198
IL-1β	1·00	0·89	0·85	0·24	13·56	7·36	10·14	2·40	0·275	<0·001	0·317
IL-6	1·00[a]	0·55	0·74[a]	0·14	74·62[c]	37·74	36·77[b]	23·28	0·048	<0·001	0·051
IL-8	1·00[a]	0·42	1·92[a]	0·55	15·37[c]	6·32	10·95[b]	2·09	0·214	<0·001	0·065
IL-10	1·00	0·52	1·13	0·56	1·31	0·56	1·35	0·27	0·722	<0·001	0·852
CCL2	1·00[a]	0·26	2·40[a]	0·83	16·11[b]	1·83	21·89[c]	1·34	<0·001	<0·001	0·001
CXCL16	1·00	0·39	1·40	0·39	2·89	0·55	2·76	0·49	0·504	<0·001	0·213
COX2	1·00	0·63	1·27	0·53	8·05	7·22	4·55	2·53	0·317	0·004	0·244

LBP, LPS-binding protein; *CD14*, cluster of differentiation factor 14; *MyD88*, myeloid differentiation factor 88; *IRAK1*, IL-1 receptor-associated kinase 1; *TRAF6*, TNF-α receptor-associated factor 6; *RIPK2*, receptor-interacting serine/threonine-protein kinase; *CCL2*, CC chemokine ligand 2; *COX2*, cyclo-oxygenase 2; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
* All the data were obtained using RT-PCR. *GAPDH* was the endogenous reference gene, and the pigs fed the maize oil diet and injected with saline were the calibrator sample.

CXCL16 and cyclo-oxygenase 2 in both gastrocnemius muscle and LD muscle ($P < 0·05$). Flaxseed oil supplementation decreased mRNA expression of LBP and CD14 in both gastrocnemius muscle and LD muscle in LPS-challenged piglets ($P < 0·05$). In addition, in both saline-injected and LPS-challenged piglets, flaxseed oil inclusion reduced mRNA expression of TNF-α receptor-associated factor (TRAF) 6, NOD2 and RIPK2 in gastrocnemius muscle, and myeloid differentiation factor 88, IL-1 receptor-associated kinase 1 (IRAK1), TRAF6, RIPK2, NF-κB and TNF-*α* in LD muscle, and increased mRNA expression of IL-10 in gastrocnemius muscle and CCL2 in both gastrocnemius muscle and LD muscle ($P < 0·05$). There was an interaction between LPS and diet for LBP, CD14 and CCL2 in both gastrocnemius muscle and LD muscle, and IL-1*β*, IL-6 and cyclo-oxygenase 2 mRNA expression in gastrocnemius muscle, and TLR4, NOD2 and IL-6 mRNA expression in LD muscle ($P < 0·05$). These variables had no difference between maize oil and flaxseed oil treatment in piglets injected with saline; however, they were all lowered after flaxseed oil was supplemented in piglets challenged with LPS ($P < 0·05$).

mRNA expression of protein kinase B1/Forkhead box O signalling and their target genes in muscles

As shown in Table 7, there was no interaction observed between LPS challenge and diet for Akt1/FOXO signalling and their target genes. LPS challenge increased the mRNA expression of FOXO1, MuRF1 and PDK4 in both gastrocnemius muscle and LD muscle ($P < 0·05$); however, these variables were all reduced in both

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Table 7. Effects of flaxseed oil or maize oil supplementation on mRNA expression of protein kinase B1 (*Akt1*)/Forkhead box O (*FOXO*) signalling pathways and its target genes after 4 h *Escherichia coli* lipopolysaccharide (LPS) challenge in weaned piglets*
(Mean values and standard deviations, *n* 6 (one pig per pen))

	Saline				LPS				*P*		
	Maize oil		Flaxseed oil		Maize oil		Flaxseed oil				
Item	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Diet	LPS	Interaction
Gastrocnemius muscle											
Akt1	1·00	0·09	0·92	0·26	0·81	0·47	0·93	0·21	0·858	0·454	0·392
FOXO1	1·00	0·16	0·71	0·27	1·75	0·32	1·33	0·44	0·012	<0·001	0·616
FOXO4	1·00	0·13	0·99	0·33	1·10	0·21	0·87	0·18	0·196	0·899	0·241
MAFbx	1·00	0·37	0·47	0·39	1·10	0·49	0·64	0·12	0·004	0·375	0·819
MuRF1	1·00	0·29	0·79	0·56	2·87	0·61	2·06	0·33	0·015	<0·001	0·134
PDK4	1·00	0·52	0·42	0·22	2·73	0·65	2·41	0·49	0·038	<0·001	0·516
Longissimus dorsi muscle											
Akt1	1·00	0·19	0·84	0·11	0·92	0·27	0·96	0·19	0·480	0·808	0·217
FOXO1	1·00	0·14	0·67	0.13	2·74	0·82	2·10	0·55	0·028	<0·001	0·464
FOXO4	1·00	0·23	0·93	0·28	1·00	0·22	1·10	0·26	0·871	0·439	0·412
MAFbx	1·00	0·40	0·86	0·56	1·01	0·15	0·94	0·23	0·493	0·751	0·793
MuRF1	1·00	0·24	0·57	0·19	3·86	0·62	3·12	1·19	0·051	<0·001	0·594
PDK4	1·00	0·26	0·53	0·28	4·20	0·68	3·53	0·67	0·013	<0·001	0·648

MAFbx, muscle atrophy F-box; *MuRF1*, muscle RING finger 1; *PDK4*, pyruvate dehydrogenase kinase 4; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.
* All the data were obtained using RT-PCR. *GAPDH* was the endogenous reference gene, and the pigs fed the maize oil diet and injected with saline were the calibrator sample.

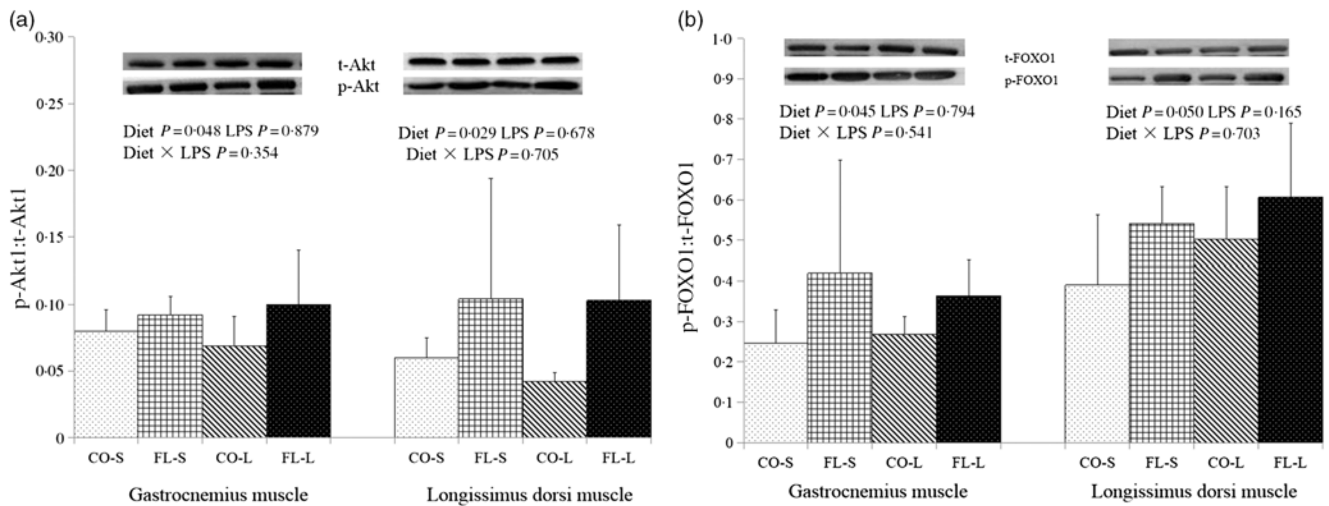

Fig. 1. Effects of flaxseed oil or maize oil supplementation on the ratios of phosphorylated protein kinase B1(p-Akt1):total Akt1(t-Akt1) (a) and phosphorylated Forkhead box O (p-FOXO1):total FOXO1 (t-FOXO1) (b) in gastrocnemius muscle and longissimus dorsi muscle in weaned piglets after *Escherichia coli* lipopolysaccharide (LPS) challenge. Values are means (*n* 6; one piglet per pen), with their standard errors represented by vertical bars. (□) CO-S, piglets fed a maize oil diet and injected with sterile saline; (▦) FL-S, piglets fed a flaxseed oil diet and injected with sterile saline; (▧) CO-L, piglets fed a maize oil diet and injected with LPS; (■) FL-L, piglets fed a flaxseed oil diet and injected with LPS. Piglets feed flaxseed oil had higher ratios of p-Akt1:t-Akt1 and p-FOXO1:t-FOXO1 in gastrocnemius and longissimus dorsi muscles in both saline-treated and LPS-challenged piglets ($P < 0·05$).

gastrocnemius muscle and LD muscle when flaxseed oil was supplemented ($P < 0·05$).

Protein phosphorylation of protein kinase B1 and Forkhead box O1 in muscles

As shown in Fig. 1, there were no interactions between LPS challenge and diet for the p-Akt1:total Akt1 (Fig. 1(a)) and p-FOXO1:t-FOXO1 (Fig. 1(b)). Flaxseed supplementation increased the ratios of p-Akt1:total Akt1 and p-FOXO1:t-FOXO1 in gastrocnemius and LD muscles in both saline-treated and LPS-challenged piglets ($P < 0·05$).

Discussion

Our previous study had found that fish oil (rich in EPA and DHA) attenuated muscle protein loss induced by LPS challenge in weaned piglets[7]. Turvey *et al.* found diet rich in *n*-3 fatty acids decreased PDK activity in human skeletal muscle[12]. Flaxseed oil, as a traditional edible oil, is rich in ALA, which is the metabolic precursor of EPA and DHA[15]. Therefore, we speculated that flaxseed oil not only had the same function in muscle protein loss as fish oil but also played an important role in attenuating carbohydrate oxidation impairment. In our study, flaxseed oil inclusion increased ALA, EPA and total *n*-6:*n*-3 PUFA contents in

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muscles, which was in agreement with the report of Zhu *et al.*[20]. In addition, Duan *et al.* reported the optimal *n*-6:*n*-3 PUFA ratios of 1:1 and 5:1 exerted beneficial effects on inflammatory system[35]. In the present study, we found that flaxseed oil supplementation could optimise this ratio compared with maize oil.

Muscle atrophy is generally associated with excessive loss of muscle protein[36]. Plasma BUN concentration is an indirect index to reflect muscle protein degradation[37] and may be useful as an indicator of protein status to quantify N utilisation and excretion rates[38]. In our study, flaxseed oil inclusion decreased BUN content in blood, suggesting that flaxseed oil attenuated LPS-induced muscle atrophy by reducing protein degradation in both normal and stress status. Muscle protein and DNA concentrations are important indexes for muscle mass or muscle protein metabolism[11]. Their ratio can be a sensitive measure for muscle protein mass[1]. In the present study, flaxseed oil inclusion prevented the reduction of protein:DNA ratio after LPS challenge, indicating that flaxseed oil could increase muscle protein mass to prevent muscle atrophy induced by LPS. This result is also consistent with our previous study in fish oil[7].

Muscle atrophy has been shown to occur concomitantly with carbohydrate oxidation impairment[1]. Glycogen in skeletal muscle can mainly supply energy for muscle contraction, and its level is associated with exogenous carbohydrate oxidation to energy expenditure[39]. Increased glycogen breakdown indicates an impairment of pyruvate oxidation[1]. In our study, flaxseed oil increased muscle glycogen content in saline-treated piglets, suggesting flaxseed oil inhibited muscle glycogen degradation in the normal status. In addition, the increased lactate accumulation is coincided with the impairment of muscle carbohydrate metabolism[1]. Lactate is mainly produced in anaerobic glycolysis. Alamdari *et al.* reported that LPS infusion attributed to muscle lactate accumulation because of the reduced PDC activation[40]. However, unexpectedly, we found flaxseed oil elevated lactate content in muscles after LPS challenge and we could not explain this result in the present study. PDC mainly controls carbohydrate oxidation by catalysing the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA in skeletal muscle and plays an important role in maintaining metabolic flexibility in skeletal muscle[41]. Moreover, PDC activation can promote immunometabolic homeostasis during sepsis[42]. PDK4 expression has an inverse relationship with PDC activity[43]. In the present study, flaxseed oil inclusion reduced PDK4 mRNA expression, indicating that this oil could increase PDC activity, which might result from the increased PDC quantity.

Pro-inflammatory cytokines, both in the circulation and locally at the level of the skeletal muscle, play a critical role in muscle atrophy and carbohydrate metabolism impairment. Previous study reported that ALA could inhibit soluble adhesion molecules release in human umbilical vein endothelial cells challenged by LPS *in vitro*[44]. LBP catalyses the transfer of LPS to CD14. Inhibiting the formation of LPS/LBP/CD14 complexes can protect the host from LPS-induced impairment[45]. In the present study, we found flaxseed oil decreased LBP and CD14 mRNA expression in muscles. TLR4 is a critical component of innate immunity[46], and it can initiate NF-κB activation through myeloid differentiation factor 88/receptor-associated kinase 1/TRAF6 signalling. Activation of NF-κB then triggers the expression of pro-inflammatory cytokines. In addition, NOD1 and NOD2 also result in NF-κB activation via RIPK2 and stimulates the release of pro-inflammatory cytokines. In our study, consistent with improved muscle protein mass (increased protein:DNA ratio) and carbohydrate metabolism (increased PDC quantity), flaxseed oil decreased plasma TNF-α, IL-6 and IL-8 concentrations and muscle TNF-α, IL-6, IL-1β, and TLR4, NOD2 and their downstream signalling molecules mRNA expression after LPS injection, which was in line with the previous reports in intestine[20]. Moreover, as an anti-inflammatory cytokine, IL-10 can prevent TLR-induced inflammatory cytokine production[47,48] and down-regulates the expression of IL-1, IL-6, IL-8 and TNF-α[49]. CCL2 is essential to mount an adequate inflammatory response to repair acute skeletal muscle injury[50]. In our study, we found flaxseed oil inclusion could increase IL-10 and CCL2 mRNA expression in muscles. These results indicated that flaxseed oil could attenuate inflammatory response through blocking the formation of LPS/LBP/CD14 complexes, inhibiting TLR4 and NOD signalling pathways and simultaneously stimulating IL-10 and CCL2 mRNA expression, which further alleviate muscle atrophy and carbohydrate oxidation impairment induced by LPS challenge.

Pro-inflammatory cytokines can lead to muscle protein loss directly or via alterations of the Akt/FOXO/ubiquitin-proteasome pathway. Akt/FOXO is a common signalling pathway influencing muscle protein breakdown and synthesis during inflammation[51]. Phosphorylation and inactivation of FOXO1 induced by Akt1 activation can suppress muscle proteolysis and muscle atrophy by transcriptional inhibition of FOXO target genes such as MAFbx and MuRF1; the latter are the main regulators in protein degradation in skeletal muscle[52]. Various pathological and physiological conditions can activate these two ubiquitin-ligase enzymes and stimulate muscle proteolysis. In our previous study, we found diet supplemented with fish oil decreased FOXO 1 mRNA abundance and increased phosphorylation of Akt and FOXO1 in muscles. In line with these results, in the present study, we also found flaxseed oil inclusion decreased mRNA expression of FOXO1, MAFbx and MuRF1 and elevated the ratios of p-Akt1:total Akt1 and p-FOXO1:t-FOXO1 in muscles, indicating flaxseed oil could have a positive effect on alleviating muscle protein loss through regulating the Akt/FOXO1/ubiquitin-proteasome pathway signalling pathway.

FOXO signalling has a dual role in both ubiquitin-mediated proteolysis and carbohydrate oxidation impairment. Crossland *et al.* reported that FOXO could impair carbohydrate oxidation *in vivo* during sepsis[53]. Mallinson *et al.* found increased FOXO activity could up-regulate PDK gene expression, which stimulated PDC phosphorylation and then depressed this complex activity[4]. PDK4 is a major determinant of PDK activity and is mainly present in muscles[54,55]. In our study, flaxseed oil inclusion reduced PDK4 mRNA expression, which indicates that this oil could improve carbohydrate metabolism to produce more energy for relieving stress induced by LPS.

In addition, fatty acid composition in diet has an effect on the long-term regulation of skeletal muscle PDK[56]. Jucker *et al.* found safflower oil could reduce glycolytic and oxidative disposal of glucose compared with fish oil[57]. Long-term sucrose-rich diet decreased PDC activity in rats; however, fish oil

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supplementation could reverse this alteration[58]. Stephens *et al.* found *n*-6 PUFA partially replaced with *n*-3 PUFA increased muscle PDC activation[59]. In our study, flaxseed oil inclusion increased PDC quantity, which might result from the rich contents of *n*-3 PUFA in flaxseed oil evidenced by the high *n*-3 PUFA content in muscle.

Previous studies have shown that proinflammatory cytokines can elevate catabolic hormones such as cortisol and glucagon[60,61]. Cortisol is an inhibitor of muscle protein synthesis through binding to its glucocorticoid receptor, and both glucagon and cortisol can increase protein breakdown and urea formation[62,63]. In addition, these hormones can also affect carbohydrate metabolism[64,65]. Glucocorticoid and glucagon could increase PDK4 mRNA expression[64,65]. However, glucagon is not generally considered to act in skeletal muscle[66], which indicates glucagon might have an indirect effect on protein metabolism in muscle. In our study, flaxseed oil reduced plasma cortisol content, indicating that flaxseed oil could attenuate protein degradation through decreasing plasma cortisol content after LPS challenge.

As we know, dynamic changes occur in protein metabolism, carbohydrate oxidation, pro-inflammatory cytokine gene expression and protein phosphorylation of signalling molecules in muscles after LPS challenge[67–69]. So, measurements taken only at one time point (4 h) are probably not adequate to confirm the roles of signalling molecules and pro-inflammatory mediators in LPS-induced muscle atrophy and carbohydrate oxidation impairment. In addition, besides TLR4 and NOD signalling pathways, many other pathways can induce the production of proinflammatory cytokines. Thus, in future studies, sample will be collected at more time points to investigate the dynamic interplay of flaxseed oil supplementation on muscle atrophy and carbohydrate oxidation. In addition, many other inflammatory pathways should also be investigated.

In summary, diet supplemented with flaxseed oil might have a positive effect on alleviating muscle protein loss and carbohydrates oxidation impairment. These beneficial effects of flaxseed oil on muscles might be associated with regulating the TLR4/NOD and Akt/FOXO signalling pathways. The present study provides a good reference for human nutrition, and flaxseed oil may have a positive role in human health.

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The authors declare no conflicts of interest.

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