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Influences of dietary flavonoid (quercetin) supplementation on growth performance and immune response of growing pigs challenged with *Escherichia coli* lipopolysaccharide

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Abstract

This study was conducted to evaluate the effects of dietary supplementation of plant flavonoid (quercetin) on immune parameters, growth performance, and nutrient digestibility in growing pigs challenged with *Escherichia coli* lipopolysaccharide (LPS). A total of 40 crossbred ([Landrace × Yorkshire] × Duroc) growing pigs; initial body weight (BW) of 26.95 ± 1.26 kg were used in a six-week experimental trial. Pigs were randomly allocated into one of four treatment groups in a 2 × 2 factorial arrangement with the following factors; without LPS challenge and with LPS challenge (day 21) supplemented with or without 0.1% flavonoid according to BW (2 replicate pens per treatment with 2 gilts and 3 barrows per pen). The single-dose LPS (100 ug / kg BW) injection showed trends tended to be increased in interleukin-6 (IL-6) after 2 h and 6 h of challenge compared with unchallenged pigs. However, other measured immune indices (white blood cell, immunoglobulin G, lymphocyte, and tumor necrosis factor), growth performance, and nutrient digestibility were not significantly different between challenged and non-challenged animals. The supplementation of flavonoid significantly increased ($p < 0.05$) average daily gain (ADG) during day 0–21, tended to increase dry matter and nitrogen digestibility, significantly reduced IL-6, increased Ig-G and WBC concentrations and increased lymphocytes percentage regardless of LPS challenge.

Keywords: Flavonoid, Growing pigs, Immune stimulation, Lipopolysaccharide

INTRODUCTION

Pigs raised in the intensive farming program inevitably face several environmental threats that impair homeostasis and increase the likelihood of infectious disease outbreaks. As a result, livestock health and productivity are negatively affected. The magnitude of challenges faced by the animal determines their ability to maintain homeostasis in order to enhance growth, productivity, and health status. Alternative solutions to the management of infectious diseases endemic to commercially farmed pigs, in addition to the use of therapeutics, must be pursued. One of the alternative approaches is to supplement the animal diet with phytobiotics which is considered to be the safest alternatives.

Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Park JH, Kim IH.
Data curation:, Park JH, Sureshkumar S.
Formal analysis: Park JH.
Investigation: Park JH, Shanmugam S, Kim IH.
Writing - original draft: Park JH, Sureshkumar S.
Writing - review & editing: Park JH, Kim IH.

Ethics approval and consent to participate
The experiment protocols used in the study were approved by the Animal Care and Used Committee of Dankook University (Ethics Approval Number: DK-2-1832).

Plant extracts from different types of plants have a wide range of bioactive compounds that have beneficial health effects [1]. Flavonoids are part of the polyphenol family of phytonutrients and each kind of flavonoid has its distinct range of acts and benefits [2]. They are mostly found in flowers, fruits, vegetables and have beneficial antioxidant effects [3]. In the last few years, a considerable number of studies focused on the effect of flavonoids, such as *Spiraea japonica* flower are rutin, quercetin, isorhamnetin, genistein and kaempferol, in improving the growth performance and health of livestock [4–6]. Quercetin, the primary flavonoid compound in this study, is one of the most known and characterized flavonoids and is the most common dietary flavonoid [7]. Recently considerable attention has been paid to flavonoid research because of the reported potential beneficial effects on human health which includes anti-inflammatory, antitumor, antiviral, anti-allergic, antiplatelet, antioxidant and immunomodulatory effects [3]. Research studies on laboratory animals have indicated that plant flavonoids inhibited the proliferation of pathogenic micro-organisms thereby enhancing intestinal efficiency, attenuated *Escherichia coli* lipopolysaccharide (LPS) and enhanced immune responses [8,9]. However, there have been few studies responses to growing pigs with a flavonoid (quercetin) under the LPS challenge. Thus, in the present study, our objectives were to evaluate the induction of immune responses with single-dose LPS challenge as well as to evaluate the effect of dietary flavonoid (quercetin) supplementation on the performance and immune responses in both LPS challenged and non-challenged growing pigs.

MATERIALS AND METHODS

The present experiment was performed at the swine experimental unit of Dankook University (Cheonan, Korea). The Animal Care and Use Committee of Dankook University approved the protocol for the current experiment.

Experimental design, animals, housing and diets

A total of 40 mixed-sex growing pigs ([Landrace × Yorkshire] × Duroc) with an initial body weight (BW) of 26.95 ± 1.26 kg (mean ± SE) were used in a 6-week experimental trial. growing pigs were blocked based on body weight to a 2 × 2 factorial arrangement with the respective factors being 1) normal saline or *E. coli* LPS injection using a syringe and bluntend catheter 2) Basal diet without flavonoid and basal diet + 0.1% plant flavonoid (quercetin) the dietary supplementation of flavonoid (quercetin) as the major compound was commercially available (Synergen, Bucheon, Korea). Each treatment consisted of 10 replicate pens with 1 pig per pen. The diets were formulated to meet or exceed [10] NRC, 2012 recommendations for all nutrients (Table 1). The stock solution of bacterial LPS (*E. coli*, 0111: B4, Sigma, St. Louis, MO, USA) was prepared by dissolving LPS in 0.9% normal saline to make a concentration of 1 mg/mL and then stored at –20℃ until its use. Twenty pigs were challenged with a single-dose of 100 μg/kg BW intraperitoneal injection of *E. coli* LPS at day 21 during the 6-week experiment. Remaining 20 pigs were intraperitoneally injected with an equal amount of sterilized normal saline (0.9% NaCl) which served as a control. Every pig was individually identified by using tags, and pigs were offered *ad libitum* feed and water, through a self-feeder and nipple drinker respectively.

Sampling and measurements

The measurement of BW was done on day 0, day 21, day 22 and day 42. To calculate the average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio (G:F), the consumption of feed was recorded on a pen basis during the experiment.

To calculate apparent dry matter (DM), nitrogen (N), and energy digestibility, 0.20% chromium

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Table 1. Composition of growing pig diets (as fed-basis)

Ingredients (%)	
Corn	73.99
Soybean meal	21.31
Tallow	1.78
Tri calcium phosphate	1.24
Limestone	0.75
Salt	0.2
Methionine (99%)	0.06
Lysine	0.42
Mineral mix[1)]	0.1
Vitamin mix[2)]	0.12
Choline (25%)	0.03
Calculated value	
ME (kcal/kg)	3,300
Crude protein (%)	16.5
Crude fat (%)	4.64
Crude fiber (%)	2.51
Lysine (%)	1.12
Methionine (%)	0.32
Calcium (%)	0.66
Phosphorous (%)	0.56
Ash (%)	4.52

[1)]Provided per kg diet: Fe, 138 mg as ferrous sulfate; Cu, 84 mg as copper sulfate; Mn, 24 mg as manganese oxide; Zn, 72 mg as zinc oxide; I, 0.6 mg as potassium iodide; and Se, 0.36 mg as sodium selenite.

[2)]Provided per kilograms of diet: vitamin A, 15,600 IU; vitamin D_3, 2,040 IU; vitamin E, 72 IU; vitamin K_3, 6 mg; vitamin B_1, 5.04 mg; vitamin B_2, 22.8 mg; vitamin B_6, 8.04 mg; vitamin B_{12}, 0.06 mg; biotin, 0.408 mg; folic acid, 2.52 mg; niacin, 66 mg; D-calcium pantothenate, 54 mg.

oxide was added to the diet as an indigestible marker for 7 days prior to fecal collection at 6th week. Fecal samples were collected randomly from at least 2 pigs (1 barrow and 1gilt) from each pen, mixed and pooled and a representative sample was stored in a freezer at −20℃ until analyzed. All feed and fecal samples were freeze-dried and finely ground to pass through a 1 mm screen. DM and N digestibility were determined using methods established by the Association of Official Analytical Chemists [11]. UV absorption spectrophotometry (UV-1201, Shimadzu, Kyoto, Japan) was used to determine chromium in the diets and feces and Parr 6100 oxygen bomb calorimeter (Parr Instrument, Moline, IL, USA) was used to analyze energy by measuring the heat of combustion in the samples. For the calculation of apparent total tract digestibility following formula was used: digestibility (%) = (1 − [{Nf × Cd} / {Nd × Cf}]) × 100, where Nf = nutrient concentration in faeces (% DM), Nd = nutrient concentration in diet (% DM), Cd = chromium concentration in diet (% DM), and Cf = chromium concentration in faeces (% DM). The rectal temperature of all animals was recorded at 0 h, 2 h, 6 h, and 12 h after LPS or saline injection using a digital thermometer (Center 375 RTD Thermometer, Center technology, Taipei, Taiwan).

For blood profile analysis, 6 pigs per treatment (3 pigs/pen), whose initial body weights were close to the median weight, were randomly selected and bled via jugular venipuncture at 0, 2, 6, and 12 hours after *E. coli* LPS or saline injection during day 21 of the experiment. To obtain serum and whole blood we collected blood samples into vacuum tubes containing no additive and tubes containing K3EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) respectively.

An automatic blood analyzer (ADVIA 120, Bayer, Tarrytown, NY, USA) was used to analyze white blood cell (WBC) counts and lymphocyte from whole blood samples. The lymphocytes were then expressed as a percentage of the total WBC. The serum was centrifuged for 15 min at 3,000×g at 4℃, and stored at −4℃ until the determination of serum immunoglobulin G (IgG), tumor necrosis factor-alpha (TNF-α), (R and D Porcine ELISA kit, R and D Systems, Minneapolis, MN, USA), and interleukin 6 (IL-6) (R and D Porcine IL-6 ELISA kit, R and D Systems).

Statistical analysis

Data were subjected to two-way analysis of variance (ANOVA) according to the factorial design of the study using SAS (SAS Inst., Cary, NC, USA). The experimental unit for this experiment was pen. The SEM was used to express variability in data Tukey's test was used to determine the differences among the treatment means. Probability level of $p < 0.05$ was considered a statistically significant and $p < 0.1$ was considered tendency.

RESULTS

The effect of flavonoid (quercetin) on blood metabolites of growing pigs challenged with LPS is presented in Table 2. The concentration of IL-6 tended to be increased at 2 h ($p = 0.077$) and 6 h

Table 2. Effect of flavonoid on blood profile in LPS challenged growing pig

Items	Saline		LPS		SEM	*p*-value		
	−Fla	+Fla	−Fla	+Fla		LPS effect	Flavonoid effect	Interaction
0 h								
WBC (10^3/μL)	14.73	14.47	18.50	16.85	0.50	0.4500	0.1483	0.5856
Lymphocyte (%)	34.34	34.93	38.87	37.39	2.05	0.4944	0.0987	0.3381
IgG (10^3/μL)	273	280	315	308	17	0.7645	0.1203	0.5572
TNF-α (%)	140.4	114.1	144.0	105.0	11.1	0.8355	0.0234	0.6376
IL6 (%)	80.1	68.4	87.9	77.0	4.9	0.0987	0.0271	0.9281
2 h								
WBC (10^3/μL)	14.79	17.66	14.76	16.63	0.65	0.4054	0.0012	0.4380
Lymphocyte (%)	39.17	41.98	43.08	42.45	2.21	0.3797	0.6590	0.4884
IgG (10^3/μL)	280	340	281	330	19	0.8435	0.0229	0.8203
TNF-α (%)	111.3	103.8	107.1	97.9	10.1	0.6908	0.5120	0.9438
IL6 (%)	83.6	70.2	88.7	80.9	4.6	0.0777	0.0221	0.5207
6 h								
WBC (10^3/μL)	18.20	17.40	17.02	17.65	0.60	0.4178	0.8844	0.2190
Lymphocyte (%)	46.16	48.76	46.77	48.09	0.88	0.9739	0.0379	0.4762
IgG (10^3/μL)	251	318	245	291	16	0.3076	0.0023	0.5383
TNF-α (%)	78.7	72.8	82.2	71.1	6.0	0.8908	0.2118	0.6937
IL6 (%)	75.0	65.3	80.1	73.1	3.9	0.0994	0.0355	0.7181
12 h								
WBC (10^3/μL)	18.93	18.00	17.65	18.33	0.59	0.3824	0.8129	0.1439
Lymphocyte (%)	46.70	48.66	46.69	48.77	0.85	0.9500	0.0242	0.9437
IgG (10^3/μL)	234	271	237	264	14	0.8739	0.0284	0.7055
TNF-α (%)	68.2	63.3	71.9	63.9	5.6	0.7278	0.2998	0.7999
IL6 (%)	67.4	65.9	70.5	67.6	3.9	0.5544	0.5956	0.8688

LPS, lipopolysachharide; −Fla, without flavonoid; +Fla, with 0.1% flavonoid.

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(p = 0.09) post LPS injection in challenged animals compared with the non-challenged pigs. Other immune markers such as WBC, lymphocyte, IgG, TNF-α, were not significantly different between LPS challenged and non-challenged animals. The dietary supplementation of flavonoid (quercetin) showed a significant reduction ($p < 0.05$) on IL6 concentration at 0, 2, and 6 hours post LPS injection. In addition, lymphocyte concentration was significantly improved ($p < 0.05$) during 6 and 12 h, and also, IgG concentration was higher ($p < 0.05$) during 2, 6, and 12 h post LPS injections in quercetin supplemented groups compared with non-supplemented pigs regardless of LPS challenge. The rectal temperature was within the normal range in both LPS challenged and non-challenged animals with or without the inclusion of dietary flavonoid (quercetin) (Table 3). The supplementation of quercetin resulted in higher ($p < 0.05$) ADG but the BW, ADFI; G/F remained unaffected during day 0–21. The BW, ADG, ADFI, G/F during day 22–42 as well as overall experiment period was also not affected significantly. LPS challenge did not significantly affect the growth performance parameters as compared with control (Table 4). The apparent total tract digestibility (ATTD) of DM and N tended to be higher in pigs receiving flavonoid supplemented diet than those without flavonoid supplemented diet, but ATTD of energy remained unaffected in pigs fed flavonoid

Table 3. Flavonoid on rectal temperature in LPS challenged growing pig

Items	Saline		LPS		SEM	*p*-value		
	−Fla	+Fla	−Fla	+Fla		LPS effect	Flavonoid effect	Interaction
Temperature (℃)								
0 h	39.75	39.93	40.17	39.67	0.37	0.8394	0.6877	0.4101
2 h	39.95	40.02	40.31	39.85	0.17	0.6047	0.3137	0.1927
6 h	39.84	39.98	39.86	39.79	0.16	0.5054	0.8270	0.6349
12 h	39.81	39.82	39.57	39.62	0.18	0.3662	0.9177	0.9177

LPS, lipopolysachharide; −Fla, without flavonoid; +Fla, with 0.1% flavonoid.

Table 4. Effect of flavonoid on growth performance in LPS challenged growing pig

Items	Saline (CON)		LPS		SEM	*p*-value		
	−Fla	+ Fla	−Fla	+ Fla		LPS effect	Flavonoid effect	Interaction
Body weight (kg)								
Initial	26.96	26.95	26.94	26.94	1.01	0.9926	1.0000	0.9963
21 d	40.96	41.49	41.04	41.47	0.93	0.9779	0.6327	0.9619
22 d	41.04	41.38	40.65	41.40	0.87	0.8415	0.5638	0.8247
Finish	55.64	56.19	55.36	56.15	1.12	0.8954	0.8519	0.9152
0–21 d								
ADG (g)	667	693	671	692	8	0.8646	0.0465	0.7346
ADFI (g)	1,290	1,276	1,283	1,281	27	0.9719	0.7929	0.8470
G/F	0.517	0.543	0.523	0.541	0.016	0.8837	0.2513	0.8154
22 d-finish								
ADG (g)	730	740	735	737	20	0.9541	0.7747	0.8452
ADFI (g)	1,803	1,856	1,802	1,841	30	0.8084	0.2107	0.8318
G/F	0.405	0.399	0.408	0.401	0.010	0.8343	0.8397	0.9442
Overall								
ADG (g)	683	696	684	695	8	1.0000	0.2087	0.9067
ADFI (g)	1,503	1,522	1,499	1,517	10	0.6827	0.1319	0.9806
G/F	0.454	0.458	0.456	0.458	0.005	0.7994	0.6506	0.8785

LPS, lipopolysaccharide; −Fla, without flavonoid; +Fla, with 0.1% flavonoid; ADG, average daily gain; ADFI, average daily feed intake; G/F, gain to feed ratio.

Table 5. Effect of flavonoid on nutrient digestibility in LPS challenged growing pig

Items (%)	Saline		LPS		SEM	*p*-value		
	−Fla	+Fla	−Fla	+Fla		LPS effect	Flavonoid effect	Interaction
Dry matter	70.42	71.97	69.64	71.79	0.70	0.5326	0.0572	0.6927
Nitrogen	68.92	70.19	69.47	70.61	0.56	0.4374	0.0972	0.9165
Energy	70.35	71.87	69.50	71.30	1.91	0.6190	0.2755	0.9206

LPS, lipopolysachharide; −Fla, without flavonoid; +Fla, with 0.1% flavonoid.

supplemented diet. There was no significant difference in nutrient digestibility between controls or LPS challenged animals (Table 5).

DISCUSSION

A wide range of physiological responses is generally expected to be provoked due to inflammatory challenges. The responses to immune challenges include, fever, reduced feed intake, changes in cytokines concentrations, increase in plasma concentration of acute-phase proteins as well as hypothalamic-pituitary-adrenal axis activation [12–15]. In the current study, *E. coli* LPS challenge was chosen to induce the stimulation of the immune system in pigs kept under commercial conditions and to evaluate the dietary effects of plant flavonoid on immune-related indices, rectal temperature and growth performance of growing pigs challenged with or without LPS.

A study by Wright et al. [14] performed that the stimulation of immune response lasted for less than 24 h with single-dose LPS administration. Therefore, we evaluated the immune indices and body temperature at 0, 2, 6, and 12 h after single-dose LPS injection at day 21 of the experiment. Results from the present study indicated that intraperitoneal administration of LPS to growing pigs did not have a significant effect on body temperature as well as pro-inflammatory cytokine, and immune-related indices such as WBC count, lymphocyte percentage, IgG, and TNF-α concentration; although a trend in increase in IL-6 concentration was observed after 0, 2, and 6 h of LPS injection.

In a review, Tanaka et al. [16] noted that infections and tissue injuries induce acute phase responses as a host defense mechanism resulting in IL-6 production. The trend in the increased concentration in IL-6 after 2 and 6 h of LPS challenge in the present study indicates that the LPS challenge had a very mild effect in stimulating the acute phase immune response. In contrast to the present finding, elevation in body temperature following the intraperitoneal administration of 100 μg/kg BW LPS in weaned pigs has been reported Wright et al. [14] & Johnson & Bore [12]. In addition, increased plasma TNF-α concentration increased WBC counts and reduction in lymphocyte percentage has been reported with LPS challenge [17,18]. Similarly, LPS injection has been reported to induce the production of IgG [19]. Previous studies also noted that LPS immune challenge caused growth reduction by depression in feed intake [14,20] which is inconsistent with the present study findings. The failure to observe the significant immune stimulation response as well as reduced growth performance in the present study may probably due to a single-dose LPS challenge since some studies noted that immune stimulation is activated by multiple low-dose LPS treatment Upadhaya et al. [21] & Zhong et al. [18] or it may be due to the route of LPS injection administration, presence of protein impurities in LPS preparation or variation in serotypes used in different studies [22,23]. Although E.coli LPS O111:B4 is the most frequently used in porcine research Williams et al. [24]; however, the presence of protein impurities in those LPS preparations may affect the immune stimulation responses [25,26]. Moreover, it has been reported that WHO still accepts a 50% to 200% variation from the mean value in the well-accepted Limulus amebocyte lysate test [27].

In the earlier study, Li et al. [28] stated that *in vivo* animal studies with quercetin were found to

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demonstrate an anti-inflammatory effect. It has been shown that quercetin inhibits LPS-induced TNF-α production in macrophages and block IL-6 secretion [29,30]. In the present study also, it was found that supplementation of quercetin to the diet of pigs' significantly reduced IL-6 concentration at 2, 6, and 12 h after LPS/saline injection. In addition, WBC counts, IgG concentration and lymphocyte percentage were positively influenced in the present study by quercetin supplementation regardless of LPS challenge. Quercetin acts mainly on leukocytes and targets many enzymes and membrane proteins, intracellular signaling kinases and phosphatases, which are often crucial for a cellular specific function thereby affecting immunity and inflammation. For instance, LPS-induced inflammation is reduced by inhibiting Src- and Syk-mediated phosphatidylinositol-3-kinase (PI3K)- (p85) tyrosine phosphorylation [31].

In poultry study, Liu et al. [32] demonstrated that that intermediate levels (0.2–0.4 g/kg feed) of quercetin supplementation improved feed efficiency in hens, but higher levels (0.6 g/kg feed) had a negative effect. Similarly, Goliomytis et al. [33] found relatively high inclusion levels caused a non-significant increase in the feed conversion ratio (FCR) of broiler chickens. In the present study, except for an increase in ADG during day 0–21, none of the other growth performance parameters were affected by 0.1% quercetin supplementation in growing pigs. A trend in increase in the DM and N digestibility with quercetin supplementation seen in the present study might be due to the alteration of the intestinal microbiota resulting in lower systemic inflammation and improved metabolic outcomes [34]. A tendency in nutrient digestibility might had a positive effect on ADG before the LPS challenge but no effect on ADG after LPS challenge was observed. The inconsistent findings among different studies on growth performance might probably due to the dose of quercetin, animal species as well as diet types. There were no interactive effects between LPS injection and flavonoid (quercetin) supplementation in any of the measured parameters.

CONCLUSION

Taken together, there was short term mild stimulation of inflammation by single-dose LPS challenge which was indicated by a trend in the increase in plasma IL-6 concentration at 2 and 6 h after LPS injection, although other measured immune-related parameters were not triggered. The supplementation of quercetin to the diet enhanced immune function regardless of LPS challenge by reducing IL-6 concentration and increasing Ig-G, WBC and lymphocytes. The digestibility of DM and N tended to increase that might have increased the ADG of non-challenged animals. Therefore, further investigations are needed to evaluate the purified LPS injection and the supplementation of flavonoid (quercetin) at different doses to the LPS challenged animals.

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