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Effects of β-Glucan from *Paenibacillus polymyxa* and L-theanine on Growth Performance and Immunomodulation in Weanling Piglets*

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ABSTRACT : Forty weanling piglets (5.6±0.5 kg and 26 to 30 d of age) were used in a 28-d experiment to determine the effects of β-glucan from *Paenibacillus polymyxa* and L-theanine on growth performance. Piglets were randomly allotted to four groups (n = 10, 2 animals per pen) provided with the basal feed (control), β-glucan 400 mg/kg feed, L-theanine 80 mg/kg feed or β-glucan plus l-theanine (combination of the above-mentioned concentrations). Body weight and feed consumption were recorded during four weeks. Subsequently, the immunomodulatory effects of β-glucan and L-theanine were investigated for lipopolysaccharide (LPS)-induced cytokine production *in vitro* and *in vivo* on day 28. Although there were no significant differences in the growth performances among the treatment groups, β-glucan plus L-theanine had 5.6% greater ADG ($p = 0.074$) on day 21 to 28. β-Glucan alone or plus L-theanine increased interleukin (IL)-10 levels and decreased interferon (IFN)-γ and tumor necrosis factor (TNF)-α levels in cultured medium by LPS treatment ($p<0.05$). Plasma IL-10 levels were also increased in the piglets fed with β-glucan alone or plus L-theanine after LPS challenge (25 μg/kg, i.p.), whereas plasma IFN-γ and TNF-α levels were decreased ($p<0.05$). The levels of IFN-γ in piglets fed with β-glucan plus L-theanine showed the greatest inhibition after LPS challenges. In conclusion, treatment of β-glucan alone or plus L-theanine might lessen inflammatory responses against Gram-negative bacterial infection via the inhibition of pro-inflammatory cytokine production and enhancement of anti-inflammatory cytokine production. Further studies are needed to determine an optimal concentration of β-glucan and L-theanine for improved growth performance. (**Key Words :** β-Glucan, L-theanine, Piglets, Lipopolysaccharide, Cytokine)

INTRODUCTION

Weaning, a major stress factor in piglets, causes low feed consumption, reduced growth rate and increased incidence of diarrhea via morphological and physiological damage of intestine and bacterial infections (Gatnau, 1999; Beaulieu et al., 2006). To overcome these problems during the weaning periods, immunostimulating and anti-stress substances are paid attention in pig nutrient and management.

β-Glucan from *Paenibacillus polymyxa* is a β-1,6-glucan branched β-1,3-glucan (Jung et al., 2007). Several advantages of this β-glucan are low cost, high yields and easy purification due to release of produced β-glucan from the bacteria in medium. β-Glucans as biological response modifiers, exhibited host-mediated antitumor activity and stimulation of reticulo-endothelial system such as macrophage cells (Suzuki et al., 1990; Kraus and Franz, 1991; Adachi et al., 1994). These immunomodulatory activities improved animal health status through enhancement of host immune system against pathogenic microbial infections (Kiho et al., 1998; McIntosh et al., 2005).

L-theanine (γ-glutamylethylamide), a unique amino acid from *Camellia sinensis*, has various biological activities, including anti-hypertensive, neuroprotective and anti-oxidant effects (Yokogoshi et al., 1995; Yokozawa and

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Table 1. Dietary composition of the diets (as-fed basis)

Ingredient	% of diet
Corn	44.95
Soybean meal (48% CP)	20.00
Dried whey	20.00
Fish meal	5.00
Cornstarch [a]	2.00
Wheat bran	2.00
Soybean oil	3.00
Monocalcium phosphate	1.22
Limestone	0.50
Salt	0.35
Vitamin premix[b]	0.30
Trace mineral premix[b]	0.15
L-lys·HCl	0.15
DL-methionine	0.38
Calculated nutritive value	
CP (%)	20.64
Ca (%)	0.81
P (%)	0.63

[a] β-Glucan replaced cornstarch in the experimental treatments.

[b] Vitamin and trace mineral premix provided per kilogram of complete diet: vitamin A, 11,000 IU; cholecalciferol, 5,000 IU; vitamin E, 70 IU; vitamin K3, 4 mg; riboflavin, 6 mg; D-pantothenic acid, 20 mg; niacin, 30 mg; Mn from $MnSO_4 \cdot 5H_2O$, 20 mg; Zn from $ZnSO_4 \cdot 7H_2O$, 50 mg; Fe from $FeSO_4 \cdot H_2O$, 150 mg; Cu from $CuSO_4 \cdot 5H_2O$, 120 mg; I from KI, 0.3 mg; and Se from $Na_2SeO_3 \cdot 5H_2O$, 0.25 mg.

Dong, 1997; Kakuda et al., 2002). Oral intake of L-theanine increased α-brain wave activity in human and hence induction of relaxation (Juneja, 1999). In addition, Kimura et al. (2007) reported that oral intake of L-theanine could cause anti-stress effects via inhibition of cortical neuron excitation.

Immunomodulatory activities of β-glucan and anti-oxidant effects of L-theanine may improve general performances and the host immune systems in piglets during weaning periods. In these studies, we evaluated the effect of β-glucan from *Paenibacillus polymyxa* and L-theanine dietary supplementation on growth performance and immunomodulation in weaned piglets.

MATERIALS AND METHODS

Materials

β-Glucan (90%) and L-theanine were kindly given from Bio Industry Center (Daegu, South Korea) and Dongbu Fine Chemical (Seoul, South Korea). LPS from *Escherichia coli* serotype O55: B5, trypan blue and Histopaque 1.077 were obtained from Sigma-Aldrich Chemical Inc (MO, USA). RPMI 1640 and fetal calf serum (FCS) was purchased from Invitrogen (NY, USA) and BioWittaker (MD, USA). Swine tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-10 ELISA kits were obtained from Biosource (CA, USA).

Animals and experimental design

All animal procedures were approved by the Institutional Animal Care and Use Committee of Chungnam National University. Forty Yorkshire piglets (5.6±0.5 kg and 26 to 30 d of age) were used. The pigs were allotted into 20 pens (2 piglets/pen) according to their initial body weight. The pens were randomly divided to 5 pens of four dietary treatments (10 piglets/treatment group): basal feed (control), β-glucan (400 mg/kg feed), L-theanine 80 mg/kg feed and β-glucan plus l-theanine (combination of the above-mentioned concentration). Piglets were provided *ad libitum* access to feed and water. In Table 1, the diets were formulated to meet or exceed the requirements for all nutrients (NRC, 1998). Piglets were weighed and feed consumption was measured weekly to determine the average daily gain (ADG), average daily feed intake (ADFI), and gain:feed (G:F) ratio for each group during 4 weeks.

On 28 day, blood samples were collected from five piglets of each group by jugular venipuncture to evaluate the effects of β-glucan in peripheral blood mononuclear cells (PBMCs). Collected samples were separated by gradient density centrifugation using Histopaque 1.077. PBMCs were washed twice with RPMI 1640 medium, counted, and seeded at 1×10^6/ml in 24-well microplate after detection of cell activity with trypan blue dye exclusion. The RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin. The PBMCs were incubated at 37°C in 5% CO_2 atmosphere for 24 h. PBMCs in suspension (1×10^6 cells/ml) were incubated with or without LPS at a final concentration of 10 μg/ml for 3, 6, 12 and 24 h. After incubation, cell-free supernatants were collected and stored at -20°C until the analysis for TNF-α, IL-10 and IFN-γ.

To determine immunomodulatory activities of β-glucan in weaned piglets challenged with LPS, another 5 piglets of each group were intraperitonially injected with 25 μg/kg LPS on 28 day. The LPS was dissolved in a saline solution. Blood samples were taken at 0, 3 and 6 h after LPS injection, and plasma was separated by centrifugation (2,000 g for 20 min) for analysis of TNF-α, IL-10 and IFN-γ.

Determination of cytokines

Concentrations of TNF-α, IFN-γ and IL-10 in plasma were determined by commercial swine ELISA kits (Biosource, CA, USA). The sensitivity thresholds were <3.0 pg/ml for TNF-α, <2.0 pg/ml for INF-γ and <3.0 pg/ml for IL-10. Samples were assayed in triplicate and the assays were analyzed colorimetrically using a microplate reader.

Statistical analysis

All data of growth performances and cytokine responses were analyzed by ANOVA with GLM procedures of SPSS

Table 2. Effect of β-glucan and L-theanine on growth performance in weanling piglets

Parameters[a]	Control[b]	β-glucan	L-theanine	Combination[b]	SEM
		400 mg/kg feed	80 mg/kg feed		
Day 0 to 7					
ADG (g)	206	215	221	220	12
ADFI (g)	350	330	320	340	17
G:F ratio	0.59	0.652	0.691	0.647	0.024
Day 7 to 14					
ADG (g)	394	412	404	407	14
ADFI(g)	523	518	516	524	22
G:F ratio	0.753	0.796	0.783	0.777	0.031
Day 14 to 21					
ADG (g)	526	532	557	552	14
ADFI (g)	722	732	748	724	18
G:F ratio	0.729	0.728	0.745	0.763	0.052
Day 21 to 28					
ADG (g)	601	618	623	634	22
ADFI (g)	956	951	957	954	27
G:F ratio	0.629	0.65	0.651	0.665	0.046
Day 0 to 28					
ADG (g)	432	457	451	448	14
ADFI (g)	638	633	635	644	19
G:F ratio	0.677	0.692	0.71	0.696	0.034

[a] ADG = Average daily gain; ADFI = Average daily feed intake; G:F ratio = Gain:feed ratio.

[b] Control, supplemented with basal feed; Combination, supplemented both β-glucan 400 mg/kg feed and L-theanine 80 mg/kg feed.

ver. 12.0 (SPSS In., IL, USA), using diet treatment as a classification factor. Pen was used as the experimental unit for growth performance data, whereas individual piglet was used as the experimental unit for cytokine responses. In cytokines responses analysis, *post hoc* comparisons of each treatment groups to control treatment within each time-point were performed using Tukey's test. The alpha level used for determination of significance was $p<0.05$, with statistical tendencies reported when $p<0.10$ (Nofrarías et al., 2006).

RESULTS

Effects of β-glucan on the growth performance in piglets

The results of growth performance during the experimental period are presented in Table 2. There were no differences for ADFI and G:F ratio among dietary supplement with β-glucan and L-theanine. From day 21 to 28, piglets fed β-glucan plus L-theanine had greater ADG ($p = 0.074$), compared with that of animals fed basal feed. β-Glucan only supplement resulted in a greater ADG ($p = 0.097$) on day 0 to 28.

Effects of β-glucan on the cytokine production from PBMCs

Immunomodulatory effects of β-glucan and L-theanine in PBMCs were described in Table 3. There were not any changes for the productions of the cytokines in PBMCs incubated without LPS (data not shown), whereas LPS treatment increased TNF-α and IFN-γ levels in culture supernatant in all time points except 0 h ($p<0.05$). β-Glucan only or plus L-theanine dietary supplement significantly decreased TNF-α in cultured medium at 12 and 24 h after lipopolysaccharide treatment ($p<0.01$). Piglets treated with β-glucan only or plus L-theanine showed lower concentration of IFN-γ in culture supernatant at 12 and 24 h than control ($p<0.05$ and $p<0.01$, respectively). In addition, β-glucan only or plus L-theanine dietary supplement showed higher IL-10 concentrations at 24 h in culture supernatant than basal feed ($p<0.05$). L-Theanine only supplement showed no significant difference in all parameters, comparing with control.

Effects of β-glucan on the cytokine production in weanling piglets challenged with LPS

As shown in Table 4, β-glucan alone or plus L-theanine dietary supplement significantly decreased plasma TNF-α level at 3 to 6 h after LPS challenge ($p<0.05$). Plasma IFN-γ level of the piglets supplemented with β-glucan alone or plus L-theanine was lower than control at 3 and 6 h after LPS challenge ($p<0.01$ and $p<0.05$, respectively). The treatment of β-glucan plus L-theanine showed the greater inhibition of IFN-γ than that of β-glucan alone. β-Glucan alone or plus L-theanine increased the concentration of plasma IL-10 at 3 h after LPS challenge ($p<0.05$). L-Theanine alone dietary supplement did not show significant difference in all estimated parameters, compared with control.

Table 3. Effect of dietary β-glucan and L-theanine on the cytokine production by peripheral blood mononuclear cells stimulated with lipopolysaccharide (*Escherichia coli* O55:B5, 10 μg/ml)

Parameters[a]	Control[b]	β-glucan	L-theanine	Combination[b]	SEM
		400 mg/kg feed	80 mg/kg feed		
TNF-α (ng/ml)					
0 h	1.24	1.17	1.39	1.14	0.03
3 h	1.83	1.58	1.75	1.66	0.06
6 h	2.35	1.90	2.12	1.87	0.07
12 h	2.40	1.87**	2.26	1.74**	0.13
24 h	2.13	1.64**	1.90	1.62**	0.07
IFN-γ (pg/ml)					
0 h	3.11	3.58	2.23	2.24	0.33
3 h	45.34	41.27	47.85	42.35	1.49
6 h	73.18	63.23	71.24	61.26	2.94
12 h	71.36	59.69*	69.27	56.12*	3.68
24 h	68.22	53.21**	64.54	52.49**	3.99
IL-10 (pg/ml)					
0 h	34.57	34.23	35.73	35.12	0.33
3 h	35.29	37.25	36.58	37.54	0.51
6 h	37.24	43.22	39.49	44.37	1.65
12 h	39.22	45.69	42.11	47.38	1.81
24 h	44.35	52.67*	45.33	53.69*	2.42

[a] TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL-10, interleukin-10.

[b] Control, the basal feed; Combination, supplemented with both β-glucan 400 mg/kg feed and L-theanine 80 mg/kg feed.

*,** Means are different from control within each time-point, $p<0.05$ and 0.01, respectively.

DISCUSSION

In the present study, β-glucan from *Paenibacillus polymyxa* and L-theanine were first investigated to improve growth performance in weanling piglets. Performance data demonstrated no significant improvement in growth performances in Table 1. However, β-glucan alone and plus L-theanine had 5.6 and 5.5% greater ADG ($p = 0.097$ and $p = 0.074$, respectively). 1,3-β-Glucan dietary from *Saccharomyces ceriviciae* supplement was related to decrease ADFI, with no effects on ADG and G:F ratio (Dritz et al., 1995). 1,3-1,6-β-Glucan extracted from *Saccharomyces ceriviciae* showed increased ADG (Eicher et al., 2006). Mao et al. (2005) reported that dietary supplement of 1,3-1,6-β-glucan from Chinese herb *Astragalus membranaceus* did not show improvement of

Table 4. Effect of β-glucan on the cytokine production in piglets (n = 5) challenged intraperitonially with lipopolysaccharide (*Escherichia coli* O55:B5, 25 μg/kg)

Parameters[a]	Control[b]	β-glucan	L-theanine	Combination[b]	SEM
		400 mg/kg feed	80 mg/kg feed		
TNF-α (ng/ml)					
0 h	0.41	0.37	0.38	0.34	0.01
3 h	2.93	2.25**	2.72	1.80**	0.27
6 h	1.22	0.96*	1.05	0.82*	0.05
9 h	0.54	0.41	0.51	0.35	0.06
IFN-γ (pg/ml)					
0 h	4.62	3.23	5.60	4.11	0.52
3 h	238.34	192.97**	226.64	175.32**	14.61
6 h	106.36	82.40*	102.13	94.23	12.25
9 h	78.25	71.30	75.42	68.35	9.19
IL-10 (pg/ml)					
0 h	55.49	53.63	50.65	54.37	1.03
3 h	87.56	102.10*	90.63	104.38*	9.25
6 h	83.24	90.54	81.47	91.21	7.50
9 h	61.30	73.62	67.46	75.22	7.24

[a] TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL-10, interleukin-10.

[b] Control, the basal feed; Combination, supplemented with both β-glucan 400 mg/kg feed and L-theanine 80 mg/kg feed.

*,** Means are different from control within each time-point, $p<0.05$ and 0.01, respectively.

entire performances. These finding suggested that β-glucans from various sources were able to cause divergent responses in relation with their structures and sources.

LPS is a major component of the cell wall of gram-negative bacteria and leads to septic shock and endotoxemia (Cirioni et al., 2002). In the pathogenesis of bacterial infection, it shows various toxic effects and inductions of pro-inflammatory cytokines in host cells, such as TNF-α, and IL-6 (Carroll et al., 2002; Touchette et al., 2002). The hyperproductions of pro-inflammatory cytokines play a critical role in the pathophysiology of bacterial and viral infections (Spurlock et al., 1997). Specially, TNF-α causes hypotension, myocardial suppression, and multiple organ failures immediately after bacterial infection and inflammatory stimuli (Knotek et al., 2001). Nakajima et al. (1995) reported that repetitive inoculation of LPS induced fibrinoid vasculitis, meningoencephalitis and pneumonitis in piglet. Severity of the clinical symptoms is related to the amount of endogenous TNF-α. In the current study, β-glucan alone or in combination with L-theanine dietary supplement significantly decreased TNF-α levels in the cultured medium of PBMC and plasma. These results imply that β-glucan from *Paenibacillus polymyxa* enables to prevent the hyperproduction of pro-inflammatory cytokines against gram-negative bacteria infections.

IL-10, one of anti-inflammatory cytokines, prevents macrophage secretion of TNF-α and IL-12 and thereby inhibits IFN-γ production of activated natural killer cell (Fiorentino et al., 1991). Moreover, Fuirebtubi et al. (1989) and De Waal Malegyt et al. (1991) found that IL-10 could inhibit T-cell proliferation and the secretion of both Th1- and Th2-type cytokines. IFN-γ, produced by T lymphocytes and natural killer cells, is a macrophage-activating factor (Carroll et al., 2003). Activated macrophages by IFN-γ produce pro-inflammatory cytokines, reactive oxygen radicals and nitric oxide to remove pathogenic microorganism. However, overproduction of nitric oxide by activated macrophages is also able to cause undesirable pathological status (Cauwels and Brouckaert, 2007; Rastaldo et al., 2007). Furthermore, IL-10 can also suppress the signal transduction of the nuclear transcription factor κB, which is a major transcription factor of proinflammatory cytokines (Clarke et al., 1998; Li et al., 2006a). In the present studies, β-glucan and β-glucan plus L-theanine revealed lower IFN-γ levels *in vitro* and *in vivo* after LPS exposure than control, while enhancing higher production of IL-10 production. Taken together, β-glucan may cause reduced hyperproduction of IFN-γ, following increased production of IL-10 in the time dependent manner.

In weanling piglets, many researchers reported the improvements of growth performance via the enforcement of the immune system by β-glucan (Dritz et al., 1995; Mao et al., 2005; Li et al., 2006b). β-Glucan inhibited the overproduction of proinflammatory cytokines, such as TNF-α and IFN-γ, inducing production of anti-inflammatory cytokines, such as IL-10. These finding is agreement with our results (Tables 3 and 4). However, the molecular weight, conformation, and branch of β-glucans might influence for their modulatory activities of inflammation-related cytokine (Kulicke et al., 1997; Yadomae, 2000). Nevertheless, excessive doses of β-glucans did not improve growth performances in weanling piglets (Schoenherr et al., 1994). β-Glucan can either induce or suppress the release of TNF-α from mononuclear phagocytes in relation with doses (Hoffman et al., 1993). Therefore, the optimal concentration of β-glucan from *Paenibacillus polymyxa* and L-theanine is needed for further validations on the improvements of growth performance. However, β-glucan 400 mg/kg feed or plus L-theanine 80 mg/kg feed may be warranted for the cytokine response in the present study.

L-Theanine extracted from green tea plants attracts its relaxing and anti-stress effects (Juneja et al., 1999; Kimura et al., 2006). In addition, L-theanine had increased resistance of bacterial infections and enhanced the secretion of IFN-γ in relation to memory response of peripheral blood T cells (Kamath et al., 2003). In our studies, L-theanine alone treatment did not showed significantly differences in growth performance and immunological responses *in vitro* and *in vivo*, compared with control. However, the levels of IFN-γ in piglets fed with β-glucan plus L-theanine showed the greatest inhibition after LPS challenges (Table 4). Because effects of L-theanine on cytokine production are not yet clearly, its action mechanism and interaction with β-glucan should be clarified.

In conclusion, the dietary supplementation of β-glucan and L-theanine in weanling piglets led to increased tendencies of ADG, together with the benefit effects of immunomodulation against LPS challenge. Therefore, further studies are needed to investigate the effects of β-glucan and L-theanine on improvements of growth performances at various concentrations for the validation.

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