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Effect of Korean red ginseng marc fermented by *Bacillus subtilis* on swine immunity

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Abstract

Red ginseng marc is a by-product of Korean red ginseng (*panax ginseng* CA Meyer) and contains ginsenoside which has pharmacological effects. The Korean red ginseng marc was fermented with *Bacillus subtilis* (RGMB). This study was carried out to investigate the RGMB effect on swine immunity. The variation of ginsenoside depending on the RGMB fermentation time was analyzed. Swine (Landrace× Yorkshire) were divided into control group (basic diet) and RGMB group (RGMB 1% diet). One percent RGMB was fed to the RGMB group for 28 days. The biochemical parameters, cytokine and immunoglobulin were analyzed. For 48 hours of fermentation on RGMB, ginsenoside Rb1 had increased 180.94%, Rg3 235.85%. Rg1 wasn't detected before fermentation, but was detected after 48 hours of fermentation. The RGMB had effect of deceasing initial AST concentration 79.33±12.85 U/L to 54.00±14.46 U/L in final and was significantly lower ($P<0.05$) than control in final. In final RGMB had significantly lower ($P<0.05$) ALT concentration of 48.57±8.26 U/L comparing with control group of 65.43±10.31 U/L. RGMB had the effect of significantly decreasing ($P<0.05$) IL-1β, IL-6 and TNF-α concentration of 2.44±1.31 ng/mL, 0.71±0.36 ng/mL and 0.51±0.21 ng/mL. The IgA concentration had significantly increased ($P<0.05$) in RGMB group of 0.56±0.06 mg/mL in final. These results demonstrate that RGMB has effect of increasing immunity and practicable to use as feed additives on swine.

Key words : *Panax ginseng, Bacillus subtilis*, Fermentation, Swine, Immunity

INTRODUCTION

In modern animal husbandry, livestock are raised in large-scale intensive operation to maximize the livestock production. However the large-scale intensive operation has increased heat and environment pollution in the farm, deteriorate livestock immunity and increased the livestock disease rate (Tilman et al, 2002; Ilea, 2009). Therefore solution to increase the livestock immunity has been requiring in the animal husbandry. In the past, antibiotic was used as a feed addition on livestock. However many countries currently prohibit or limit using antibiotics as feed addition as it has been studied the antibiotics remain in the livestock products, causing people to develop resistance toward antibiotics when they intake the livestock's products (Lee et al, 2001; Ko et al, 2007). Therefore researches of using natural substances are being actively studied as feed addition to improve livestock's immunity (Bong et al, 2011).

Korean ginseng (*Panax ginseng* CA Meyer) is a perennial plant belonging to the Family *Araliaceae*, Genus *Panax* and has been used as an important herbal medicine in the oriental medicine (Choi, 1991). Ginseng can be classified as white and red ginseng by how it was processed. White ginseng is made by drying fresh ginseng root with hot air or sun-dried, whereas red ginseng is made by steaming and drying the fresh ginseng root (Ha and Ryu, 2005). During the process of steaming red

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ginseng, chemical structure of the red ginseng changes due to the heat. The low content or non-existent of ginsenosides such as Rg2, Rg3, Rh1, Rh2 increases during the streaming process. Therefore red ginseng has its characteristic pharmacological action compared with other ginsengs (Nam, 2005). Red ginseng is known to have pharmacological actions of anticancer (Kwak et al, 2003a), antioxidant (Kim et al, 2004), blood circulation improvement (Shin et al, 2007) and infection defense (Kaneko and Nakanishi, 2004).

Red ginseng products are usually made by extracting red ginseng with water or alcohol. During this process, quantities of red ginseng by-products are created, namely red ginseng marc (Sung et al, 1985; Kwak et al, 2003b). Red ginseng marc is usually discarded, although ginsenoside and acidic polysaccharide still exist (Chang et al, 2007).

Fermenting red ginseng with microorganism increases the content of beneficial component of ginsenoside (Kim et al, 2010; Shin, 2010). The increased ginsenoside in fermented ginseng improve the immunity, antioxidant and anti-inflammatory than regular ginseng (Kim et al, 2011; Jung et al, 2012). It is studied, fermenting red ginseng marc with microorganism increases the ginsenoside and pharmacological effects (Lee et al, 2015). *Bacillus* spp. produce antibiotics against phytopathogenic fungi and also synthesize poly-c-glutamic acid (c-PGA) which is biodegradable, non-toxic and has been successfully used in food industries (Wang et al, 2008; Ogunleye et al, 2015). *Bacillus subtilis* (*B. subtilis*) is a widely used, dominant species in fermentation of food and is known to improve digestibility and absorbability (Kiers et al, 2000; Chantawannakul et al, 2002).

However, although the beneficial effect of fermented red ginseng studies, studies of red ginseng marc fermented with *B. subtilis* and researches of fermented ginseng marc effect on livestock immunity is scarce. Therefore the purpose of this study was to investigate the effect of Korean red ginseng marc fermented with *B. subtilis* (RGMB) on swine. The RGMB was mixed 1% with the basal diet and was fed to the swine for 28 days. On initial and final experiment day, biochemical parameters, cytokines and immunoglobulin concentration were investigated.

MATERIALS AND METHODS

Fermented red ginseng marc preparation

In this study, red ginseng marc fermented by *B. subtilis* (RGMB) was supplied from Han Dong CO., LTD. (Seoul, Korea). The RGMB was used in powder and liquid form. Red ginseng marc was crushed into 100 μm mesh. 2.1×10^8 CFU/mL of *B. subtilis* was inoculated into 12.5% (w/v) ginseng powder medium. RGMB was fermented for 24 hours and 48 hours with the following of pH 6.5, 36±1°C, rpm 250, dissolved oxygen (DO) 150 ppm. The sludge of RGMB was removed by centrifuging at 7,500 rpm. The 48 hours fermented RGMB was used as liquid form for *In Vivo* test. The RGMB powder was prepared by hot air dried at 55°C of 0, 24 and 48 hours fermented RGMB for ginsenoside analysis.

Analysis of ginsenoside content

Ginsenoside concentration in 0, 24, 48 hours RGMB was analysis by High Performance Liquid Chromatography (HPLC). The RGMB powder was dissolved into 50% (v/v) methanol (HPLC grade, J.T. Baker). The supernatant was filtered into 0.22 μm membrane filter. Analytical condition of HPLC was applied from standard of health functional food provide from Ministry of Food and Drug Safety (Korea). The analysis of ginsenoside content was carried out using Hitachi Primaide (Hitachi, Japan), ProntoSIL 120-5-C18 columns (250 mm×4.6 mm, German). The mobile phase was acetonitrile (HPLC grade, J.T. Baker) and water (HPLC grade, J.T. Baker). The mobile phase gradation appears in Table 1. For analysis, sample injection was 10 μL, UV detector at 203 nm, column oven 30°C and flow rate of 1.0 mL/min. Ginsenoside standard was used Rb1 (Extrasynthese Cedex, France), Rg1 (Extrasynthese Cedex, France), Rg3 (Sigma-Aldrich, USA).

Animals and treatment

Twenty swine (Landrace×Yorkshire) of 4-week-old, were divided into control group and RGMB group (ten

Table 1. Composition of mobile phase employed in the gradient HPLC system

Time (Min)	Composition of mobile phase (%)	
	Water	Acetonitrile
0	80	20
5	80	20
20	77	23
25	70	30
30	60	40
35	50	50
75	15	85
80	80	20

swine/group) and were experimented for 28 days. The control group was fed with basal diet and the RGMB group was fed with 1% (v/w) of RGMB liquid mixed with the diet. The water and diet was fed freely.

Blood collection

Blood were collected from each group on initial and finish. The blood were collected from the jugular vein and were put into BD vacutainer SST tube (BD, Cowley, Oxford, UK). The serum was separated by clotting the blood from the BD vacutainer SST tube in the room temperature and centrifuged at 2,500 rpm for 15 minutes. The serum were stored at -70^{o}C.

Biochemical parameters of serum

The biochemical parameters of serums were analysis by an automatic biochemical analyzer (Mindray BS-400, Shenzhen, China). The contents of aspartate aminotransferase (AST), alanine aminotransferase (ALT) was analyzed.

Analysis of cytokine concentration

Cytokine concentrations of interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) in serum were analyzed using Milliplex MAP swine cytokine/chemokine magnetic bead panel kit (Millipore corporation, MA, USA). The assays were performed according to the manufacturer's instruction. The plate reading was performed using Luminex 200TM (Luminex, Austin, TX, USA). The concentration of the cytokines was calculated using a standard curve.

Table 2. Changes of ginsenoside contents of RGMB on different fermentation time

Ginsenoside (μg/mL)	0 hrs RGMB	24 hrs RGMB	48 hrs RGMB
Rb1	8.97	11.76	25.2
Rg1	-	-	3.84
Rg3	15.65	52.56	49.9

- Means content not detected.

Analysis of immunoglobulin concentraion

Immunoglobulin concentration of immunoglobulin G (IgG), immunoglobulin M (IgM) and immunoglobulin A (IgA) in serum were analyzed using pig IgG, pig IgM and pig IgA ELISA kit (Komabiotech, Seoul, Korea). The assays were performed according to the manufacturer's instruction. The serums were diluted 1:20,000 using assay diluent (1% BSA). The plate reading was performed using microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA), optical absorbance at 450 nm. The concentration of the immunoglobulin was calculated using a standard curve.

Statistical analysis

The results values were expressed as mean ± standard deviation (SD). Statistical analysis among groups was performed with t-test. The statistically significant was considered $P<0.05$.

RESULTS

Analysis of ginsenoside contents

The ginsenoside Rb1, Rg1 and Rg3 contents of 0, 24, 48 hours fermentation of RGMB are presented in Table 2. Before the fermentation, 0 hrs RGMB appeared having small amounts of ginsenoside contents of Rb1 8.97 μg/mL, Rg3 15.65 μg/mL and Rg1 wasn't detected. After 48 hours of fermentation, all the analyzed ginsenoside contents had increased in RGMB. Rb1 of 48

hours RGMB was 25.2 μg/mL, increment of 180.94% and Rg3 was 49.9 μg/mL, increment of 218.85%. Rg3 had the highest increment of 235.85% at 24 hours RGMB of 52.56 μg/mL. It is assumed Rg3 content has the highest increment at 24 hours. Rg1 wasn't detected at 0 hours and 24 hours RGMB. It is presumed that 0 hours and 24 hours RGMB had none or minimum contents of Rg1 which wasn't able to be analyzed. However the fermentation of RGMB for 48 hours had increased the Rg1 content to 3.84 μg/mL.

Biochemical parameters of serum

The effect of RGMB in biochemical parameters is presented in Table 3. There wasn't any significant different of AST and ALT concentration between control group and RGMB group on initial. AST concentration of RGMB group had significantly decreased ($P<0.05$) from initial 79.33±12.85 U/L to final 54.00±14.46 U/L while control group had a similar AST concentration between initial and final. Also RGMB group AST concentration on final was significantly lower ($P<0.05$) than control group. At final, ALT concentration on RGMB group was significantly lower ($P<0.05$) than control group. ALT concentration in control group had increased from initial 45.63±5.71 U/L to final 65.43±10.31 U/L. However ALT concentration on RGMB group had a similar concentration between initial and final.

Table 3. Effect of RGMB in blood biochemical parameters concentration on swine

Parameters	Control	RGMB
AST (U/L)		
Initial	77.50±9.58	79.33±12.85
Final	79.40±13.69[a]	54.00±14.46[b]
ALT (U/L)		
Initial	45.63±5.71	46.13±6.90
Final	65.43±10.31[a]	48.57±8.26[b]

[a,b]Means in the same row with significant difference ($P<0.05$).

Table 4. Effect of RGMB in cytokine concentration on swine

Cytokine	Control	RGMB
IL-1β (ng/mL)		
Initial	7.15±0.62	7.23±0.88
Final	8.85±1.63[a]	2.44±1.31[b]
IL-6 (ng/mL)		
Initial	2.71±0.37	2.85±0.47
Final	3.24±0.50[a]	0.71±0.36[b]
TNF-α (ng/mL)		
Initial	0.75±0.35	0.76±0.36
Final	1.70±0.24[a]	0.51±0.21[b]

[a,b]Means in the same row with significant difference ($P<0.05$).

Cytokine concentration

The cytokine concentrations of IL-1β, IL-6 and TNF-α are presented in Table 4. On initial, cytokine concentration were similar between the groups and didn't have significant different. However on final, cytokine concentration of control group had increased, contrastively when cytokine concentration of RGMB group had decreased. Cytokine concentration of RGMB group were all significantly lower ($P<0.05$) than control group on final. On RGMB group, IL-1β had decreased from 7.23±0.88 to 2.44±1.31 ng/mL, IL-6 decreased from 2.85±0.47 to 0.71±0.36 ng/mL and TNF-α decreased from 0.76±0.36 to 0.51±0.21 ng/mL.

Immunoglobulin concentration

Immunoglobulin concentration of IgG, IgM and IgA are shown in Table 5. On initial, Immunoglobulin concentrations were similar between the groups and there wasn't any significant. IgG, IgM and IgA concentration in control group and RGMB group had increased on final. On final, RGMB group IgG concentration was higher than control group, whereas IgM concentration of

Table 5. Effect of RGMB in immunoglobulin concentration on swine

Immunoglobulin	Control	RGMB
IgG (mg/mL)		
Initial	4.99±0.76	4.88±1.27
Final	7.07±1.05	8.26±1.08
IgM (mg/mL)		
Initial	1.19±0.16	1.07±0.27
Final	3.26±0.77	2.95±0.54
IgA (mg/mL)		
Initial	0.20±0.04	0.20±0.05
Final	0.38±0.07[a]	0.56±0.06[b]

[a,b]Means in the same row with significant difference ($P<0.05$).

RGMB group was lower than control group. However IgG and IgM concentrations between the groups didn't have any significant difference. IgA concentration on final of RGMB group was 0.56±0.06 mg/mL, which was significantly higher ($P<0.05$) than control group IgA concentration of 0.38±0.07 mg/mL.

DISCUSSION

Korean ginseng (*Panax ginseng* CA Meyer) has been used as an important herbal medicine in the oriental medicine (Choi, 1991). Red ginseng has ginsenoside such as Rg2, Rg3, Rh1, Rh2 which non-exist in fresh ginseng (Ha and Ryu, 2005). Therefore the red ginseng has characteristic pharmacological action compared with white ginsengs and is known to increase immunity (Nam, 2005). Red ginseng marc is a by-product of red ginseng after extracting with water and alcohol (Sung et al, 1985; Kwak et al, 2003b). However it is known red ginseng marc still have ginsengoside and the same pharmacological action with red ginseng (Chang et al, 2007).

The red ginseng marc in this study was fermented for 48 hours with *B. subtilis*. This study had analysis three ginsenoside Rb1, Rg1 and Rg3 which are the standard ginsenosides of red ginseng from the health functional food provide from Ministry of Food and Drug Safety (Korea). From the result, ginsenoside Rb1, Rg1 and Rg3 had increased after the fermentation. It has had appeared similar with Kim et al. which reported fermentation of red ginseng with microorganism increases ginsenoside contents (Kim et al, 2010). The ginsenoside of RGMB had increased effectively for 48 hours appearing increment of Rb1 180.94% and Rg3 218.85%. The Rg1 wasn't detected on 0 hrs and 24 hrs RGMB, but suddenly appeared on 48 hrs RGMB. It is presumed that 0 hours and 24 hours RGMB had none or minimum contents of Rg1 which wasn't able to be analyzed. However the fermentation of RGMB for 48 hrs had increased the Rg1 content to 3.84 μg/mL. Based on these results, *B. subtilis* has a high effectiveness of increasing ginsenoside contents in red ginseng marc and the fermentation time also relate with the increase of ginsenoside contents (Kim et al, 2010).

AST and ALT are used as indexes of liver damage (Bhanwra et al, 2000) and liver related disease and mortality (Petrov et al, 2010). It has been reported fermented red ginseng had effect of decreasing AST and ALT concentration and protective effect of liver damage (Kim et al, 2011). The RGMB had shown high effect of significantly decreasing ($P<0.05$) AST concentration from 79.33±12.85 U/L to 54.00±14.46 U/L. The ALT concentration had similarly remained comparing on initial and final while the ALT concentration on control group had increased on final. Also ALT concentration of RGMB group was significantly lower ($P<0.05$) than control group on final. Therefore, it is considered RGMB has protective action toward liver disease and damage.

IL-1β, IL-6 and TNF-α are known to be pro-inflammatory cytokine which secrete in primary inflammation and also participate in growing the inflammation (Calixto et al, 2004). Therefore substance which inhibits IL-1β, IL-6 and TNF-α secrete has effect of anti-inflammation and can help increasing immunity. On final, the cytokine IL-1β, IL-6 and TNF-α of control group had increased, while the RGMB group had decreased and was significantly lower ($P<0.05$) than control group. It is presumed the uncleanness environment and fight between piglets had caused stress and minor inflammation, causing to increase the cytokine concentration on the swine. However the RGMB had effect of anti-inflammation and decreased secrete of cytokine IL-1β, IL-6 and TNF-α.

Immunoglobulin is an antibody which has heavy chain and light chain structure and is produced from the plasma cell (Martin, 1969). IgG is an index of humoral immunity (Park, 2008) and known as the most important antibody in defense effect toward infection and disease (Lee et al, 1987). IgM is an antibody produced from B cell and is the first immunoglobulin to react and increases at initial exposure to an antigen. IgA is known to have critical role in immune function in the mucous membranes and defense the body from external antigen (Hill et al, 1995). The RGMB had effect of increasing IgG and IgM but didn't have significant difference ($P<0.05$) between control group on final. However RGMB

had significantly increased ($P<0.05$) IgA comparing with the control group. Due to the result of increasing IgG, IgM and significantly increasing ($P<0.05$), it is considered RGMB have effect of increasing the immunoglobulin concentration.

CONCLUSION

In this study, it was appeared fermentation of Korean red ginseng marc with *B. subtilis* had increased the ginsenoside contents of Rb1, Rg1 and Rg3. The 48 hours fermentation was most effective on increasing the ginsenoside contents. It is presumed RGMB increase immunity based on having protective action toward liver disease and damage by decreasing the ALT and AST concentration. Also RGMB had effect of reducing pro-inflammatory cytokines concentration and increasing immunoglobulin concentration. Therefore RGMB has a potential of being used as a feed additive of increasing immunity.

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