



Effects of Fermented Soybean Meal on Immune Response of Weaned Calves with Experimentally Induced Lipopolysaccharide Challenge

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ABSTRACT : The present study was conducted to evaluate the effects of fermented soybean meal (FSBM) on the level of cortisol hormone and immune-related serum proteins in weaned calves after experimentally induced lipopolysaccharide (LPS) challenge. Holstein neonatal calves (n = 21; 8 males and 13 females, BW = 42.2±6.15 kg) were randomly allocated to one of two dietary treatments: SBM (control calf starter having soybean meal (SBM) as a main protein source) and FSBM (substitute SBM in control diet with FSBM) groups. All calves were fed milk replacer using an automatic milk-feeder according to step-down milking method and weaned at 7 weeks old. Experimental diets were given to calves *ad libitum* throughout the experimental period. For LPS challenge, all calves except negative control animals given phosphate buffered saline (PBS), were injected subcutaneously with *Salmonella typhimurium* LPS on day 7 (D7) after weaning (D0). No significant difference in growth performance and milk intake was observed between SBM and FSBM calves. Feeding FSBM diet resulted in significantly (p<0.05) higher LPS-specific IgG at D12 and D19 and LPS-specific IgA at D19 in peripheral blood. Calves fed with FSBM diet also had significantly (p<0.05) higher concentration of serum haptoglobin (Hp) at D8. Overall concentration of cortisol in FSBM group was considerably lower than that of SBM group. Results from current study indicate that FSBM may provide beneficial effects in alleviating weaning stress and enhance immune status of weaned calves. (**Key Words :** Fermented Soybean Meal, Lipopolysaccharide Challenge, Weaning Stress, Cortisol, Weaned Calves)

INTRODUCTION

Weaning remains a critical phase in domestic animal production in association with digestive disorders causing growth retardation and diarrhea. Weaning is known to heighten susceptibility of calves to a variety of infectious diseases due to the attenuation of the immune system under high stress conditions including weaning, diet changes, and group rearrangement (Hickery et al., 2003). Thus, there is a tremendous interest in finding effective dietary stress reducers and/or immune enhancers that may improve the

disease resistance in weaned calves. As a protein source, soybean meal (SBM) is a common and widely used component in farm animal diets. However, due to the existence of various anti-nutritional factors (ANFs), the application of SBM as a diet for young animals has been limited (Dunsford et al., 1989; Li et al., 1990; Jiang et al., 2000). It has been known that the fermentation process with elimination of microbes and/or reduction of ANFs will make high-quality components available to young animals and thus increase the digestibility (Feng et al., 2007; Yoo et al., 2009; Chiang et al., 2010). Fermented soybean meal (FSBM) contains a variety of important nutrients including calcium and vitamins produced during the fermentation process, which should provide functional properties, such as growth promoting effect, and enhancing effect in feed efficiency (Lee, 1998; Kim et al., 1999; Feng et al., 2007). Moreover, previous studies have shown that small-sized peptides in FSBM increased the concentration of immunoglobulins in domestic animal (Wang et al., 2003; Feng et al., 2007). Some data on the effect of FSBM in enhancing immune responses through the increase of serum

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proteins in calves have been reported (Wolfswinkel, 2009; Kim et al., 2010). Cortisol is a representative stress hormone and is secreted at high levels in response to stress. Under environmental and metabolic stress, the secretion of cortisol is associated with stress-related changes in the animal such as the down-regulation of interferon- γ (IFN- γ), interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF- α), interleukin-6 (IL-6), and interleukin-8 (IL-8) (Fingerle-Rowson et al., 2003). Haptoglobin (Hp), a hemoglobin binding protein, is a representative acute phase protein (APP) in cattle, which is stimulated by inflammatory mediators and produced by liver. It has been shown that concentration of Hp increased up to 100-1,000 folds within 24 h after inflammatory response following gram-negative bacterial infection (Chan et al., 2004).

The hypothesis of this study was that supplementation of FSBM, instead of regular SBM, might reduce the stress response against weaning and improve the production of immune function-related serum proteins against lipopolysaccharide (LPS) challenge in calves. Therefore, the current study was carried out to evaluate the effects of FSBM (fermented *A. oryzae*) in calf diet based on the level of cortisol hormone and production of immune-related serum proteins in weaned calves after LPS challenge.

MATERIALS AND METHODS

Animal, management and diet

The present experiments were carried out at Dairy Science Division, National Livestock Research Institute, Korea. All experimental procedures were reviewed and approved by the ethics committee on the use of animals in research, National Livestock Research Institute, Korea. Holstein calves (n = 21; 8 males and 13 females, mean BW = 42.2 \pm 6.15 kg) were separated from their mothers within 2 hr of birth, weighed, and moved to pens with automatic milk-feeders offering no possibility of direct contact among calves, and fed colostrum at 10% of their body weight for the first 3 days. The calves were allowed free access to a calf starter, mixed grass hay, and water from a plastic bucket. All calves were fed a milk replacer using automatic milk-feeders according to step-down milking method (Khan et al., 2007). The milk replacer was provided at the rate of 20% of body weight until 28 days of age, then this rate gradually reduced to 10% at 29 to 30 days old and fed for the remaining 21 days of the preweaning period. All calves were weaned at 7 weeks old. The ingredients and major chemical composition are shown in Table 1. Calves were randomly allocated to two experimental diet groups (FSBM group = 8 calves, 3 males and 5 females; SBM group = 8 calves, 2 males and 6 females). Two experimental diets

Table 1. Ingredient and major chemical composition of diets

Ingredient (% DM)	SBM group	FSBM ¹ group
Ground corn	42.50	45.14
Soybean meal (SBM)	16.00	
Fermented SBM (FSBM)		13.36
Rapeseed meal	2.00	2.00
Corn germ meal	4.00	4.00
Corn gluten meal	12.00	12.00
Wheat hulls	12.00	12.00
Whole soybean	3.00	3.00
Alfalfa pellet	1.50	1.50
Milk replacer	2.00	2.00
Molasses	4.00	4.00
Salt	0.20	0.20
CaCO ₃	0.25	0.25
Mixed mineral	0.46	0.46
Vitamin AD3	0.072	0.072
L-lysine	0.01	0.01
Virginiamycin	0.008	0.008
DM	86.36	86.88
TDN ²	74.33	74.42
CP	23.31	23.51
Ca	0.42	0.38
P	0.48	0.46

¹ FSBM = Fermented SBM, *A. oryzae*.

² TDN = Total digestible NFC+total digestible CP+total digestible FA \times 2.25 +total digestible NDF-7.

were given *ad libitum* throughout the experimental period. Additional Holstein calves (n = 5; 3 males and 2 females, mean BW = 44.6 \pm 7.89 kg) were assigned as a negative control group (fed SBM diet) and received phosphate buffered saline (PBS) only. FSBM calf starter diet contained fermented SBM (a commercial product produced by Gene Biotech Corp., Gongju, Chungnam, Korea) substituting SBM diet. The major chemical composition of calf starter was similar between the groups.

Feed intake and growth performance

Automatic milk-feeders were used to record the intake of milk replacer, calf starter, and forage from week 1 to week 7. Overall average body weight (BW) gain was calculated from change of BW measured at weekly basis.

LPS challenge

Each calf was injected subcutaneously with 100 ng/kg BW of *Salmonella typhimurium* LPS (Sigma-Aldrich Co., St. Louis, MO), reconstituted with non-pyrogenic PBS, on day 7 (D7) after weaning (56 days old).

Blood sampling and hematology

The blood sampling was done as shown in Figure 1. For

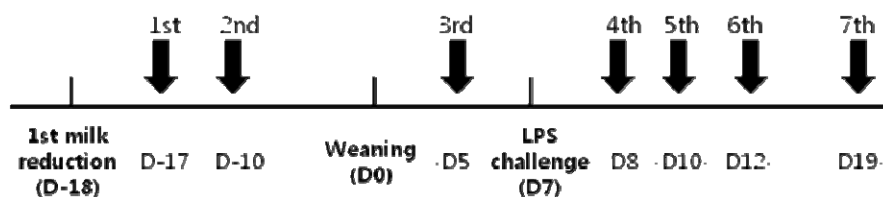


Figure 1. Blood sampling schedule during the experimental period: D0, weaning day (49 days old); D-18, 1st milk reduction (31 days old); D7, LPS challenge (56 days old). 1st milk reduction means that the supply of milk replacer started to be reduced from the rate of 20% of body weight to 10%.

the hematological test and serum proteins, 5 ml of blood was drawn from jugular vein at 54 (D5), 61 (D12) and 68 (D19) days old. Additional 10 ml of blood samples were collected into evacuated tubes coated with the anti-coagulant lithium-heparin vacutainer (BD-plymouth, PL6 7BP, UK) at 32 (D-17) and 39 (D-10) days old for cortisol assay and at 57 (D8) and 59 (D10) days old for the haptoglobin ELISA assay. Additional blood (10 ml) was collected for the ELISA and hematological assay.

After centrifugation of the blood sample at 1,600×g at 4°C for 15 min, plasma was harvested from anti-coagulated blood and stored at -80°C until further assays were conducted. Neutrophil, lymphocyte, platelet, monocyte and leukocyte population in whole blood were measured with an automatic analyzer (Hemavet 850, Drew Scientific Group Company, USA).

Enzyme-linked immunosorbent assay (ELISA)

The concentration of haptoglobin (Life Diagnostics, Inc), cortisol (Oxford Biomedical Research Inc., Oxford) and total immunoglobulins (Bethyl laboratory Montgomery, TX) was determined using an ELISA assay kit according to the procedure of the manufacturer. The concentration of

specific IgG, IgM and IgA antibodies in serum against LPS was determined as described by Trautmann et al. (1998). In brief, LPS was coated in 96-well immunoplates (Nalgene Nunc International) and incubated overnight at 4°C. Then, the plates were washed with washing buffer (0.05% Tween 20 in PBS) 3 times and blocked with washing buffer (0.05% Tween 20 in PBS) for 2 h. The plates were incubated with diluted serum samples for 3 h at room temperature and washed 3 times with washing buffer. Anti-bovine antibodies (IgG, IgA and IgM) conjugated with horseradish peroxidase (HRP) was added to the plates and incubated for 2 h. After the washing, specific binding was detected using streptavidin-HRP and tetramethylbenzidine (TMB) substrate (Sigma-Aldrich). To stop the reaction, 2 N H₂SO₄ was added to the plates. Absorbance was measured at 450 nm using a microplate reader (Molecular Devices).

Statistical analysis

All data were analyzed by ANOVA procedure for randomized complete block designs using the GLM procedures of SAS (SAS Inst. Inc., Cary, NC). Differences among means were tested using tukey procedure of SAS. Effects were considered significant at $p < 0.05$.

RESULTS

Changes of immunophysiological characteristics in negative control calves

Overall changes of immunophysiological parameters in serum from negative control calves are presented in Table 3. The level of platelet was significantly ($p < 0.05$) increased at D19 when compared to D5. No significant changes in the other hematological profiles were detected during the experimental period.

Hematology

Hematological parameters in calves before and after LPS administration are presented in Table 4. Similar to negative control group, the concentration of platelet in SBM group was significantly ($p < 0.05$) increased at D12 and D19 when compared to the value at D5. Interestingly, however, the changes on the concentration of platelet in FSBM group were not significant.

Table 2. Average body weight and milk intake in calves fed with SBM or FSBM calf starter during the experimental period

	SBM group	FSBM group
Body weight		
0 day	40.57±4.65	43.71±5.82
7 day	41.71±6.30	45.36±4.45
14 day	42.50±5.12	46.61±4.83
28 day	47.00±5.75	49.86±6.90
42 day	51.21±6.56	56.14±6.92
49 day	58.29±13.12	61.29±7.01
Milk intake		
1 week	4.86±2.95	4.75±2.66
2 week	34.21±8.21	34.11±17.45
3 week	42.86±15.94	36.65±3.03
4 week	38.36±10.38	36.08±9.80
5 week	25.99±6.22	27.69±3.33
6 week	26.65±4.57	30.93±6.34
7 week	17.45±5.30	15.56±2.82

Table 3. Changes of hematological parameters and total serum and LPS-specific immunoglobulins in calves fed with SBM diet as negative-control group at pre- and post- LPS challenge (mean±SD)

	D5 ¹	D12	D19
Hematological change			
NE ² (%)	25.81±9.30	54.58±18.82	41.52±10.42
LY ³ (%)	64.28±8.21	41.81±17.01	53.46±10.43
NE:LY	0.42±0.21	1.54±0.87	0.81±0.35
Leukocytes (10 ⁹ /L)	5.65±1.68	9.49±3.34	8.99±0.44
Platelet (10 ⁹ /L)	200.00±136.0 ^b	380.00±137.9 ^{ab}	616.00±271.5 ^a
Total serum antibodies			
IgG ⁴	17.46±4.49	24.16±14.64	24.89±12.66
IgA ⁵	59.52±9.45	53.55±35.74	80.29±5.15
IgM ⁶	0.91±0.33	0.85±0.17	0.86±0.04
LPS specific antibodies ⁷			
IgG	0.325±0.077	0.326±0.093	0.338±0.075
IgA	0.132±0.139	0.178±0.222	0.165±0.158
IgM	0.754±0.492	0.697±0.481	0.870±0.412

¹ D = Days-post weaning (the day of LPS challenge: D7).² Neutrophil. ³ Lymphocyte. ⁴ Total serum IgG concentration (mean±SD, mg/ml).⁵ Total serum IgA concentration (mean±SD, µg/ml). ⁶ Total serum IgM concentration (mean±SD, mg/ml).⁷ Relative concentrations of antigen specific antibody (mean±SD, absorbance at 450 nm).^{ab} Means with different letters in the same row differ significantly at p<0.05.**Table 4.** Hematological changes in weaned calves at pre- and post- LPS challenge (mean±SD)

		D5 ¹	D12	D19
SBM group	NE ² (%)	38.90±10.44	46.01±13.39	41.70±9.41
	LY ³ (%)	50.33±9.58	46.03±13.66	49.54±7.04
	NE:LY	0.82±0.36	1.18±0.79	0.87±0.27
	WBC ⁴	8.50±2.14	7.82±2.02	7.35±1.72
	PLT ⁵	275.00±212.62 ^b	561.50±189.04 ^a	598.83±173.19 ^a
FSBM group	NE (%)	42.10±13.53	46.49±13.74	42.87±10.85
	LY (%)	48.51±10.68	44.54±11.13	49.13±11.04
	NE:LY	0.96±0.48	1.17±0.65	0.95±0.41
	WBC	6.52±0.95	7.38±1.81	7.25±2.24
	PLT	419.83±93.21	520.83±97.08	523.14±125.67

¹ D = Days-post weaning (the day of LPS challenge: D7). ² Neutrophil. ³ Lymphocyte. ⁴ White blood cells. ⁵ Platelet.^{ab} Means with different letter in the same row differ significantly (p<0.05).

Cortisol level

Overall concentration of cortisol in FSBM group was lower than that of SBM group (Figure 2). The level of cortisol in calves fed with FSBM diet had significantly ($p<0.05$) lower than those fed with SBM diet at 1 day after milk reduction (D-17) and after LPS challenge (D8).

Total serum and antigen-specific immunoglobulin (Ig) production

Total serum IgG and IgA concentration was significantly ($p<0.05$) increased at D19 in calves fed both experimental diets when compared to those at D5 (Table 5). The concentration of LPS-specific IgG in FSBM group was significantly ($p<0.05$) higher than that of SBM group at D12 and D19 (Figure 3A). Although there was a tendency to increase in LPS-specific IgM level in both groups during the experimental period, it was not significant when compared to those at D5 (Figure 3C).

Haptoglobin (Hp) level

After LPS challenge, the significant ($p<0.05$) elevation of serum haptoglobin (Hp) was observed in FSBM group at D8 compared to D5 (Figure 4). The level of serum Hp from calves fed FSBM diet was significantly ($p<0.05$) higher than calves fed SBM diet at D8 (Figure 4).

DISCUSSION

An acute stress response can be provoked by rapid nutritional and environmental changes, for instance weaning, which may result in reduced resistance to disease and loss of normal body condition partly due to a decrease in feed intake. Therefore, effective dietary stress reducer or immune enhancer is a great interest in animal feed industry. The current study was conducted to evaluate effects of FSBM as a stress reducer based on immune responses in

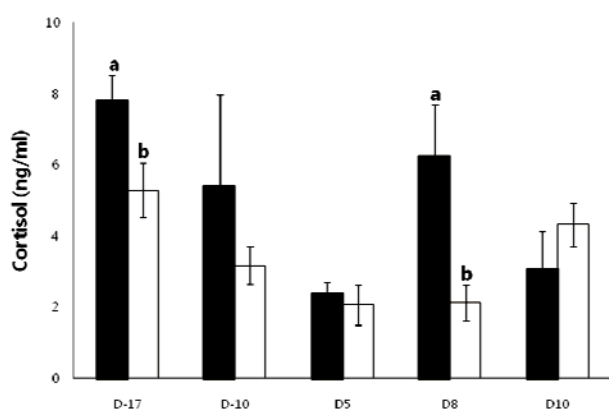


Figure 2. Change of serum cortisol level in calves fed with SBM (■) or FSBM (□) calf starter. D = Days-post weaning (the day of LPS challenge: D7). ^{ab} Means with different letters differ significantly between groups at the same time point ($p<0.05$).

Table 5. Change of total serum immunoglobulins in weaned calves fed with SBM or FSBM calf starter at pre- and post-LPS challenge (mean±SD)

	SBM	FSBM
IgG (mg/ml)		
D5 ¹	19.24±11.62	12.50±2.75
D12	24.92±18.78	15.25±3.51
D19	21.37±7.74	15.38±3.83
IgA (µg/ml)		
D5	78.96±22.43 ^b	131.73±47.47 ^b
D12	120.19±40.91 ^{ab}	153.42±59.47 ^{ab}
D19	143.02±34.33 ^a	204.28±65.39 ^a
IgM (mg/ml)		
D5	1.01±0.18 ^a	1.27±0.33 ^a
D12	1.30±0.32 ^{ab}	1.36±0.28 ^a
D19	1.59±0.51 ^b	2.34±1.21 ^b

¹ D = Days-post weaning (the day of LPS challenge: D7).

^{ab} Means with different letters in the same column differ significantly ($p<0.05$).

calves after LPS challenge following weaning.

In the current study, no difference in BW was found between treatments and milk intake in calves fed two different calf starters (Table 2). The data from calves without LPS challenge indicated that there was no time effect on immunophysiological characteristics during the experimental period. In other words, significant changes on immunophysiological parameters were not observed by natural aging during the experimental period. Therefore, it is reasonable to conclude that if any significant changes of biomarkers for immune function including hematological changes and immunoglobulin levels in serum were observed in the present study, then it would be the effects of dietary treatment and/or LPS challenge.

In the present study, the concentration of serum cortisol known as a stress marker was significantly higher in SBM group than FSBM group at one day after 1st milk reduction. Many studies have evaluated essential amino acids, such as tryptophan, glutamine, and arginine, and their impact on the response of animals to stress (Yi et al., 2005; Guzik et al., 2006; Jiang et al., 2009; Zheng et al., 2010). The results showed that feeding supplementation of amino acids reduced the cortisol response when an acute stressor including weaning or infection with microbes was introduced. Meanwhile, Chan et al. (1975) and Min et al. (2009) suggested that the FSBM might have a growth promoting activity owing to higher supply of essential amino acids and possibly vitamins synthesized during the fermentation. Therefore, lower cortisol in FSBM group in present study may also be explained in a similar manner in that FSBM could alleviate the stress response due to a greater supply of essential amino acids and possible

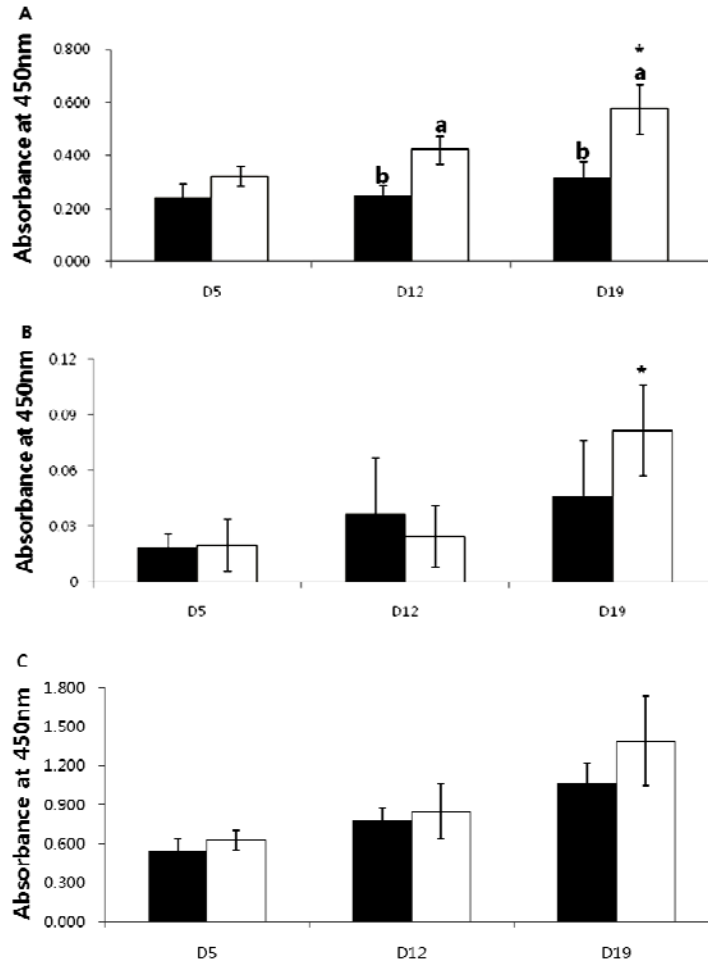


Figure 3. Change of LPS-specific (A) IgG, (B) IgA and (C) IgM in serum from calves fed with SBM (■) or FSBM (□) calf starter. D = Days-post weaning (the day of LPS challenge: D7). Means with * symbol differ significantly compared to D5 in a same group ($p < 0.05$). ^{ab} Means with different letters differ significantly between groups at the same time point ($p < 0.05$).

vitamins synthesized by the fungi fermentation. Current results also showed that total serum IgA and IgM levels significantly increased in both groups compared to those before the challenge, which may indicate that the LPS

challenge model successfully induced humoral response. It is to note that the significant increase in LPS-specific IgG and IgA levels after LPS challenge were observed in calves fed with FSBM and the level was higher than those fed with

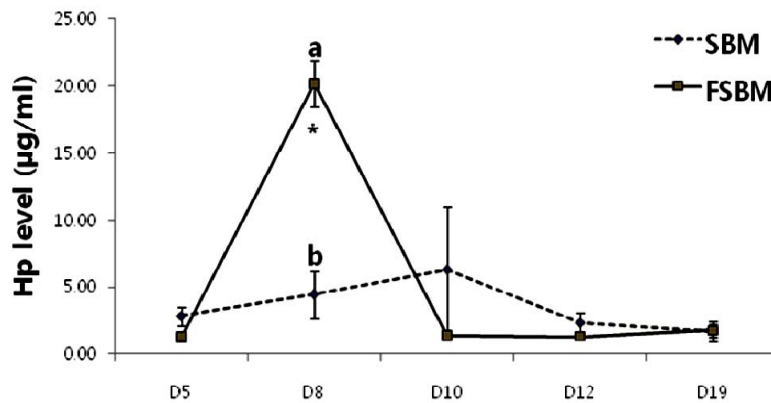


Figure 4. Changes of serum haptoglobin (Hp) level in calves fed with experimental diets after LPS challenge (◆: SBM group, ■: FSBM group). D = Days-post weaning (the day of LPS challenge: D7). Means with * symbol differ significantly compared to D5 in a same group ($p < 0.05$). ^{ab} Means with different letters differ significantly between groups at the same time point ($p < 0.05$).

SBM, indicating that calves fed FSBM diet coped with LPS challenge more efficiently. It is widely accepted that nutritional and environmental stresses enhance the secretion of cortisol, which intensely suppresses immunoglobulin production (Sabbele et al., 1983; Wiik et al., 1989; Nagae et al., 1994). Besides, during an infection with microbes, the demand for essential amino acids such as glutamine and arginine dramatically increases. Under the stressed condition, glutamine is used as a carbon source by immune cells for proliferation (Newsholme and Calder, 1997) and arginine is also required for the production of nitric oxide, a potent immunoregulatory mediator (Evoy et al., 1998). Hence, our results suggested that FSBM may have beneficial effects on attenuating stress response and enhancing B-cell response partly through supply of essential amino acids and small peptides.

In the current experimental model, subcutaneous LPS injection induced the changes in serum haptoglobin (Hp). LPS from gram-negative bacteria is known to be potent inducers of inflammation and the acute phase response, giving rise to large changes in the serum concentrations of acute phase proteins such as Hp, serum amyloid A, and albumin (Boosman et al., 1989; Werling et al., 1996). The concentration of serum Hp from our study was also significantly elevated in FSBM group in response to LPS challenge, similar to results of LPS-specific antibodies. This result implies that small sized peptides and essential amino acids in FSBM facilitated the production of acute phase protein in the same way as antibody production.

In conclusion, feeding FSBM as calf starter in calves reduced cortisol response and enhanced production of immune-related serum proteins, particularly LPS-specific IgG and IgA, and haptoglobin against LPS challenge. Therefore, FSBM may have beneficial effects on alleviating weaning stress and enhancing immune status of weaned calves.

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