

Effects of Dietary Addition of β -Glucan on Pigs Infected with *Salmonella Typhimurium*

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Abstract: Although, the antimicrobial effects of β -Glucan (BG) have been described previously, the use of β -glucan in food animal production has been unrealistic due to high production costs. Recently, pure β -glucan could be efficiently produced by cultivating mutant *Aureobasidium pullans* which contains a disrupted *pul* gene and the new method can simplify purification process and reduce mass production costs. Thus, the effect of the β -glucan purified from mutant *A. pullans* on salmonella infection in weaned pigs was evaluated in the study. Twenty, 4 weeks old pigs were randomly assigned to the following 4 groups: BG or BG-X group was provided with commercial feeds containing β -glucan (10 mg kg^{-1}) or containing both β -glucan (10 mg kg^{-1}) and Genkwadaphnin (10 mg kg^{-1}), respectively; Normal Feed (NF) and Negative control (Neg.) groups were only provided with commercial feeds only. All the groups were provided with their specific diets for 2 weeks, challenged with *Salmonella Typhimurium* at 10^8 Colony Forming Units (CFU)/mL except for the Neg. group and sacrificed for pathological evaluation at 4 weeks after the challenge. As compared with the NF group, BG and BG-X groups showed significantly lower diarrhea incidence and salmonella shedding in feces and gained more weight after salmonella infection. Gross lesions were less severe in BG and BG-X groups but the differences reached no statistical significance. In conclusion this study demonstrated that the overall performance of weaned pigs fed β -glucan derived from *A. pullan* was enhanced upon challenge with salmonella by increasing weight gains and reducing both the incidence of diarrhea and fecal shedding of salmonella.

Key words: β -glucan, genkwadaphnin, *Aureobasidium pullan*, *Salmonella Typhimurium*, weaned pigs

INTRODUCTION

Because *Salmonella Typhimurium* is one of the major serovars affecting pigs that causes a great deal of economic loss to swine industry and there are increased demands on high levels of food safety and public health, effective control and prevention against salmonella is critical (Verbrugge *et al.*, 2012). However, extensive use of Antibiotic Growth Promoters (AGPs) in food animals as a dietary additive to mitigate the risk of infections (Badia *et al.*, 2013) has raised concerns about antibiotic overuse. Antibiotic overuse in food-producing animals is considered hazardous to public health and consumers' well-being (Nunnery *et al.*, 2006). Many efforts have therefore been made to develop antimicrobial natural substances which can be substituted for conventional antibiotics. β -glucan is produced by a variety of sources such as fungi, yeast and grains, all of which have been reported to enhance host innate immunity and to have

antitumor and antimicrobial effects (Eicher *et al.*, 2006; Chen *et al.*, 2010; Li *et al.*, 2006; Badia *et al.*, 2013; Jung *et al.*, 2004). For instance, a modified variant of β -glucan purified from fungi *porio cocos* showed antitumor effects against Sarcoma 180 tumor cells in mice. In addition, it stimulated immune responses by facilitating the interactions between β -glucan and immune receptors such as complement receptor-3, toll-like receptors and dectin1 expressed on the surface of macrophage (Chen *et al.*, 2010). Moreover, β -glucans purified from yeast, *Saccharomyces cerevisiae* stimulated TNF- α production and macrophage phagocytotoxicity (Lee *et al.*, 2001). The yeast-derived β -glucan has been shown to absorb various mycotoxins and prevent bacterial adhesion to mannose-rich epithelial surface of intestines (Kogan and Kocher, 2007). β -glucan derived from oak and barley was also reported to exhibit antimicrobial activities by acidifying the intestinal tract and stimulating the growth of normal flora such as *Lactobacillus* and *Bifidobacterium* (Shen *et al.*, 2012).

Nonetheless, the use of β -glucan in food animal production would be unrealistic since it is difficult to purify β -glucan to industrial levels at a reasonable cost. The extraction and purification of β -glucan are complicated because many factors such as endogenous enzymes, solvent types, viscosity control, purity control and culture environment have to be taken into account for mass production at the bioreactor level (Crognale *et al.*, 2007). Purification of β -glucan is composed of several complicated steps which utilizes extensive use of multi-stage methods such as affinity chromatography (Lee *et al.*, 2001). However, these purification methods are generally appropriate for the laboratory-scale production which are unpractical for mass-scale production. Recently, a new method has been developed to efficiently produce a large amount of pure β -glucan using mutant *Aureobasidium pullans* which contains disrupted pullulan synthetase gene (*pul*). The mutant *A. pullans* synthesizes a higher bio-mass and production rate of β -glucan while excluding unwanted by products such as pullulan (Kang *et al.*, 2010). In this new method, β -glucan yield is increased and the purification process is simple. Thus, it reduces mass production cost of β -glucan and could facilitate the application of β -glucan for an antimicrobial feed additive in food-producing animals. Genkwadaphnin extracted from the dried flower buds of *Daphne genkwa* Sieb. Et Zucc has been used as a traditional Chinese medicine for diuretic, antitussive, expectorant and edema applications (Liou *et al.*, 1982; Hall *et al.*, 1986). Genkwadaphnin is a daphnane diterpene ester that is also known to play a major role in countering cancer by causing apoptosis in human leukemia cells and suppressing tumor in mice inoculated with Lewis lung carcinoma cells (Park *et al.*, 2007). The current study was conducted to evaluate the antimicrobial effects of β -glucan derived from mutant *A. pullulan* and to analyze its combined effects with Genkwadaphnin against salmonella infection in weaned pigs.

MATERIALS AND METHODS

Animal experiment: Twenty, 4 weeks old pigs were purchased from a pig farm without Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) swine influenza virus and mycoplasma hyopneumoniae. No history of salmonella infection has been described in the pig herd. Following a 3 days acclimation period, the pigs were randomly assigned to the following four groups:

- The BG group: the pigs were provided with commercial feeds containing β -glucan (10 mg kg⁻¹)
- The BG-X group: the pigs were provided with commercial feeds containing β -glucan (10 mg kg⁻¹) along with Genkwadaphnin (10 mg kg⁻¹)

- The Normal Feed (NF) group: the pigs were provided with commercial feeds only
- The Negative control (Neg.) group: the pigs were provided with commercial feeds only and served for a negative challenge control group

The pigs of each group were provided with specific diets until the end of the current animal experiment. At 2 weeks after the initiation of the dietary intake, the pigs of each group were orally challenged with *Salmonella Typhimurium* at 10⁸ CFU mL⁻¹ with the exception of the negative control group. Then, the fecal swabs were collected daily until 28 days after salmonella challenge and attempts were made to quantify bacterial shedding in feces. Blood samples were collected every week (at 0, 7, 14, 20, 29 and 35 days) and processed to collect serum and Peripheral Blood Mononuclear Cells (PBMC). The weight of all the pigs was measured on a weekly basis. Following this they were euthanized for necropsy and pathological evaluation at 28 days after challenge. The animal experiment protocol was approved by the Chonbuk National University Institutional Animal Care and Use Committee (Approved Number: 2012-0025).

Immunoglobulin ELISA: *Salmonella Typhimurium* specific Lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO, USA) was used as an antigen for ELISA. The LPS was prepared in 0.2 M carbonate buffer (pH = 9.4, Sigma-Aldrich) and 100 μ L of antigen was dispensed in 96 well plates. The plates were incubated overnight at Room Temperature (RT) and washed 3 times with PBST (PBS with 0.05% Tween 20). Then, the plates were filled with 300 μ L of 1% skim milk and incubated at 37°C for 1 h for blocking. Each serum sample was diluted to 1:40 with PBST and added to duplicate wells. Then, the plates were further incubated at 37°C for 1 h and washed three times with PBST. After washing, for IgG ELISA, HRP-labeled goat anti-swine IgG (Jackson Immuno Research West Grove, PA, USA) diluted to 1:10,000 with PBST was added to the wells and incubated at 37°C for 1 h. After the final washing step, Optical Density (OD) was measured at 450 nm after the color development with tetramethylbenzidine (TMB, Sigma-Aldrich) for 10 min. For IgA ELISA, biotinylated goat anti-swine IgA (Betyl Laboratories) diluted to 1:100,000 with PBST was added to wells and incubated at 4°C overnight. After washing, streptavidin-HRP (SurModics, Eden Prairie, MN, USA) diluted to 1:100,000 with PBST was added to wells and incubated further at 37°C for 1 h. After the final washing step, Optical Density (OD) was measured at 450 nm after the color development with TMB for 10 min.

Table 1: Primers used for cytokine analysis

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	References
<i>β-actin</i>	GCGGGACATCAAGGAGAAG	AGGAAGGAGGGCTGGAAGAG	U07786*
<i>TNF-α</i>	TTATTACAGGAGGCGAGGT	AGCAAAAAGGAGGCACAGAGG	NM_214022*
<i>IFN-α</i>	TCTCATGCACAGAGCCA	CCTGGACCACAGAAGGGA	Loving <i>et al.</i> (2006)
<i>IFN-β</i>	AGTGCATCCTCCAAATCGCT	GCTCATGGAAAAGAGCTGTGGT	De Los Santos <i>et al.</i> (2006)

*GenBank Accession numbers; TNF: Tumor Necrosis Factor; IFN: Interferon

Evaluation of salmonella shedding in feces: Salmonella shedding in feces was quantified by TaqMan real-time PCR. Nucleic acids were simultaneously extracted from feces using MagMax™ Total Nucleic Acid Isolation kit (Applied Biosystems, Foster city, USA) as described in the manufacturer’s manual. Briefly, 0.01 M Phosphate Buffered Saline (PBS, pH 7.4) was added to each sample to make 30% fecal homogenates. After centrifugation for 1 min at 100×g to pellet larger-size particles, 175 μL of the supernatant of each sample was added to a bead tube containing zirconia beads and 235 μL of lysis/binding solution. The bead tube was beaten at maximum speed for 5 min with Bullet Blender® (Next Advance, Averill Park, NY, USA). After the beating process, the bead tubes were centrifuged at 16,000×g for 3 min and the supernatant was carefully transferred into clean microcentrifuge tubes. After another centrifugation at 16,000×g for 6 min, 115 μL of the supernatant was transferred to a 96 well, microplate along with washing and elution buffers and the plate was placed in KingFisher® 24 magnetic particle processor for automated extraction process. The automated process consisted of lysis/binding step for 5 min; two-time first washing step each for 90 sec; two-time second washing step each for 2 min and 30 sec, respectively; dry step for 1 min and finally, the elution step for 3 min. The extracts were subjected to TaqMan based real-time PCR to determine the presence of salmonella in the samples by targeting a common Salmonella enterotoxin (*Stn*) gene (Moore and Feist, 2007; Cho *et al.*, 2010). The sequence information of primers were GCCATGCTGTTTCGATGAT (Stn-Fow) and GTTACCGA TAGCGGAAAGG (Stn-Rev) and probe was 5' FAM-TTTTGCACCACMGCCAGCCC-BHQ-3' (Stn-Pr). The real-time PCR was carried out with TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) in the 25 μL reaction volume. Samples with a threshold Cycle (Ct) = 35 cycles were considered positive for the PCR tests.

Cytokine expression: Peripheral Blood Mononuclear Cells (PBMC) were isolated by density centrifugation using Histiopaque-1077 (Sigma-Aldrich). PBMC were diluted to 1×10⁶ and cultured with *S. Typhimurium* specific LPS (10 μg mL⁻¹, Sigma-Aldrich) in 24 well plates for 24 h at 37°C. Total RNA was purified from the cultured PBMC pellets using GeneAll® Hybrid-RTM (GeneAll, Seoul, Korea) and cDNA was synthesized with random hexamers

using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). The expression levels of Interferon (IFN)-α, IFN-β and Tumor Necrosis Factor (TNF)-α were measured by 7500 Fast Realtime PCR System (Applied Biosystems) with primers listed in Table 1, using Power SYBGR® Green kit (Applied Biosystems). The levels of cytokine expression were normalized to that of β-actin expression and Relative Quantities (RQ) were determined by the 2^{-ΔΔC_T} Method.

Pathological evaluation: All pigs were humanly euthanized at 2 weeks after salmonella infection and then subjected to necropsy. Gross lesions observed in intestines were scored and intestine sections (one each from duodenum, ileum, jejunum and large intestine) were collected in 10% neutral buffered formalin and routinely processed for microscopic evaluation. Gross and microscopic lesions were evaluated in a blind fashion and then scored from 0 (normal) to 3 (severe) for severity of lesions.

Statistical analysis: All statistical analyses were conducted using SSPS (SAS Institute Inc., Cary, NC, USA). The repeated measurements of the level of salmonella shedding and antibody response in pigs were analyzed with repeated measures ANOVA to define the overall difference and pairwise comparison between viruses or groups was conducted by contrast. Non-parametric analysis, Wilcoxon test was applied to analyze lesion scores and weight gain.

RESULTS

Weight gains and clinical signs after Salmonella infection: Weight gains and clinical symptoms are summarized in Table 2, 3 and Fig. 1. The incidence of diarrhea was evaluated for 7 days after infection with *S. typhimurium*. No event of diarrhea was observed in the BG group, the BG-X group and the Neg. group. But all the pigs of the NF group exhibited diarrhea between 2 and 5 days post-challenge (dpc) two pigs showed diarrhea at 6 dpc and no diarrhea was observed at 7 dpc (Table 2). Following an analysis of the Average Daily Weight Gain (ADWG) prior to Salmonella challenge, it was slightly higher in the groups treated with no β-glucan, i.e., the NF

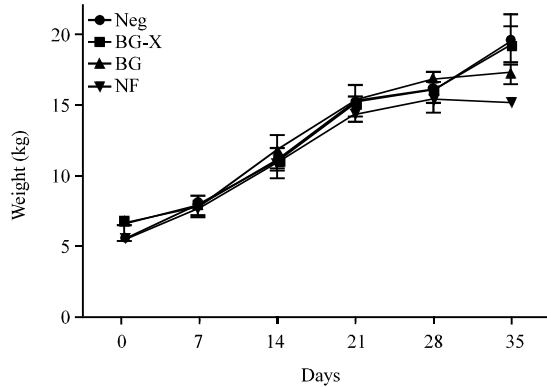


Fig. 1: Changes in body weight of pigs before and after salmonella infection. The weight of the pigs of all the four groups (BG, BG-X, NF and Neg.) was measured weekly during the experiment. All the groups except for the Neg. group were challenged with *Salmonella* Typhimurium at 10^8 CFU mL⁻¹ at 14 days

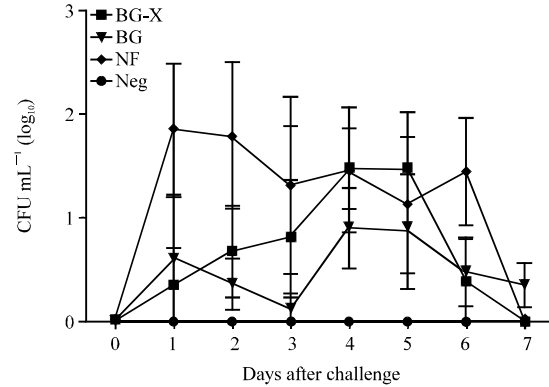


Fig. 2: The number of salmonella shedding in feces after salmonella infection. Fecal samples were collected on a daily basis until 7 days after salmonella challenge and were evaluated for salmonella shedding by real-time PCR

Table 2: Incidence of diarrhea after salmonella infection

Groups	Days after challenge						
	1	2	3	4	5	6	7
No challenge (Neg.)	0/5*	0/5	0/5	0/5	0/5	0/5	0/5
β-glucan	0/5	0/5	0/5	0/5	0/5	0/5	0/5
β-glucan-X	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Normal feed	0/5	5/5	5/5	5/5	5/5	2/5	0/5

*The number of pigs with diarrhea/The total number of pigs of each group

Table 3: The Average Daily Weight Gain (ADWG) before and after salmonella challenge

Groups	ADWG (kg day ⁻¹)	
	Before challenge ^a	After challenge ^b
No challenge (Neg.)	0.376±0.027 ^c	0.624±0.043
β-glucan	0.344±0.123	0.500±0.052
β-glucan-X	0.306±0.058	0.509±0.247
Normal feed	0.384±0.069	0.436±0.038

^aBefore challenge: 0-14 days; ^bAfter challenge: 14-28 days (after salmonella challenge at 14 days); ^cMean of average daily weight gain±SE

group and the Neg. group (0.376 and 0.384 kg day⁻¹, respectively) as compared with those treated with β-glucan, i.e., the BG group and the BG-X group (0.344 and 0.306 kg day⁻¹, respectively). After challenge with *S. Typhimurium*, however, ADWG was higher in the BG group and the BG-X group (0.500 and 0.509 kg day⁻¹, respectively) as compared with the NF group (0.436 kg day⁻¹). ADWG was the highest in the Neg. group (0.624 kg day⁻¹).

Bacterial shedding in feces after Salmonella infection:

Salmonella shedding in fecal samples were quantified by real-time PCR for 7 days after infection and summarized in Fig. 2. Salmonella was detected in feces collected at 1 dpc

from all of the challenged groups. Fecal shedding of *Salmonella* in the NF group reached its peak value of 71.3 CFU mL⁻¹ at 1 dpc and gradually decreased to 27.8 CFU mL⁻¹ at 6 dpc. No *Salmonella* shedding was detected at 7 dpc. On the other hand, *Salmonella* shedding in the BG group reached its peak value of 8.0 CFU mL⁻¹ at 4 dpc and gradually decreased to 2.2 CFU mL⁻¹ at 7 dpc. The shedding in the BG-X group reached its peak value of 30 CFU mL⁻¹ at 4 dpc and decreased to 2.4 CFU mL⁻¹ at 6 dpc. No shedding was detected at 7 dpc. The mean number of *Salmonella* shed in the NF group, the BG group or the BG-X group was 31.8, 4.0 or 10.8 CFU mL⁻¹, respectively.

Induction of Salmonella LPS-specific IgG and IgA:

The levels of IgG and IgA induction are summarized in Fig. 3. Before *Salmonella* challenge, there was a slight increase in IgG and IgA levels at 14 days. The 1 week after *Salmonella* challenge, however, there was a sharp increase at 21 days in the BG group, the BG-X group and the NF group. But there was no significant difference in the degree of increase in levels of IgG and IgA between the BG group, the BG-X group and the NF group. Moreover, the levels of IgG and IgA in the Neg. group were maintained constant throughout the evaluation period.

The difference in cytokine expression between the groups with and without the dietary addition of β-glucan:

The degree of the expression of such cytokines as IFN-α, IFN-β and TNF-α in PBMC collected at 0, 1 and 2 weeks before *Salmonella* challenge are summarized in Fig. 4. No significant cytokine expression was detected in PBMC collected up on arrival at the animal facility (0 week,

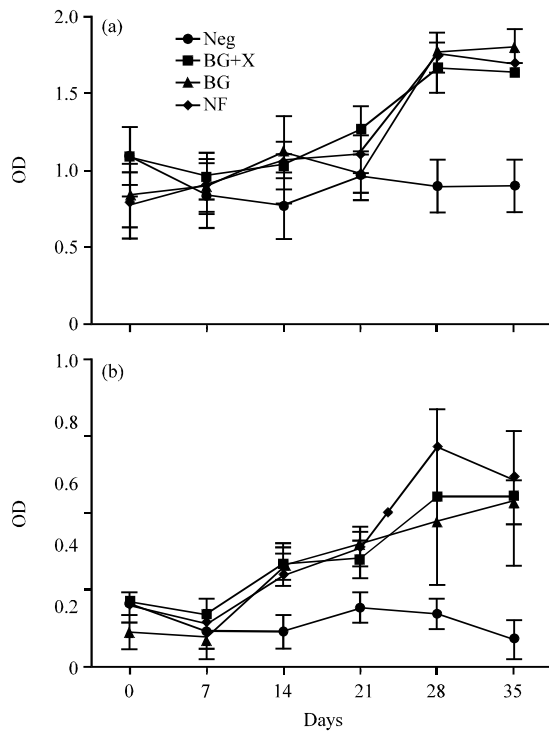


Fig. 3: The levels of; a) IgG and b) IgA specifically induced against *Salmonella Typhimurium*-specific LPS before and after salmonella infection. Serum samples were collected on a weekly basis until 35 days and analyzed with ELISA prepared with LPS purified from *Salmonella Typhimurium*

Fig. 4a). Despite a lack of the statistical significance, the degree of IFN- α expression was higher in the BG group (RQ = 4.749) and the BG-X group (RQ = 3.598) as compared with the NF group (RQ = 2.439) at 1 week after the dietary intake (Fig. 4b and c). The degree of IFN- β expression was also the highest in the BG group (RQ = 5.597) followed by the NF group (RQ = 3.043) and the BG-X group (RQ = 2.612). Similarly, the degree of the expression of IFN- α and IFN- β at 2 weeks was higher in the BG group and the BG-X group as compared with the NF group but this difference reached no statistical significance. Furthermore, there was no significant difference in the degree of TNF- α expression between the four groups.

Gross and microscopic lesions in intestines: Gross lesions were categorized into congestion, lymph node enlargement and the thickness of intestinal wall while microscopic lesions were categorized into congestion, inflammation and villous atrophy (Fig. 5). Gross lesions were generally less severe in the pigs fed with β -glucan (the BG group and the BG-X group) as compared with

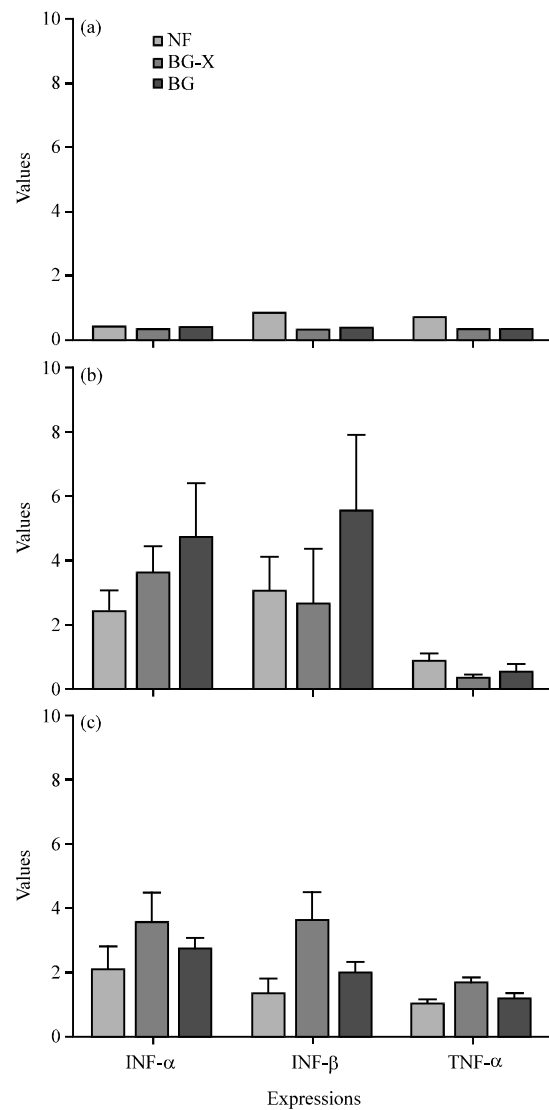


Fig. 4: The levels of cytokine expression in pigs fed with different diets. Pigs were fed with the corresponding diets for 2 weeks and Peripheral Blood Mononuclear Cells (PBMC) were collected at; a) 0 b) 1 and c) 2 weeks and cultured with *Salmonella Typhimurium*-specific LPS ($10 \mu\text{g mL}^{-1}$) for 24 h at 37°C . The levels of cytokine expression were normalized to that of β -actin expression which was expressed as Relative Quantities (RQ)

those fed with normal feeds (the NF group) although this difference reached no statistical significance. In contrast, there were no differences in microscopic lesion scores between the *Salmonella* challenge groups (the BG group, the BG-X group and the NF group). There were no notable gross and microscopic lesions in the Neg. group.

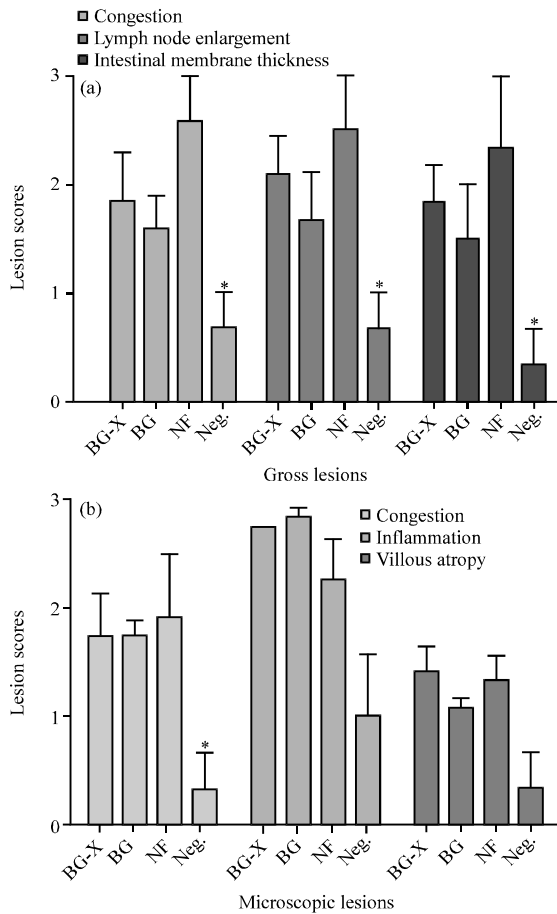


Fig. 5: The summary of gross and microscopic lesion scores. Necropsy was carried out after the pigs were humanely euthanized after salmonella challenge when intestinal samples were evaluated in a blind fashion (from 0 = normal to 3 = severe). Asterisk represents the significant difference ($p < 0.05$)

DISCUSSION

The results demonstrated that the dietary addition of β -glucan purified from mutant *A. pullans* was effective in reducing the incidence of bacterial shedding and diarrhea in pigs challenged with Salmonella. Fecal shedding of Salmonella in the groups fed with β -glucan (the BG group and the BG-X group) was 3-8 times lower as compared with the NF group. In addition, no diarrhea occurred in the BG group or the BG-X group. In the NF group, however, the pigs suffered from diarrhea from 2-5 dpc. It has been previously shown that β -glucan favors the growth of commensal gut microbial flora like Lactobacilli in weaned piglets (Pieper *et al.*, 2008) and enhances the production of Short Chain Fatty Acids (SCFA) in *in vitro* model of

porcine gastrointestinal tract (Pieper *et al.*, 2009). While the efficient growth of normal intestinal microflora could inhibit the intestinal colonization of bacterial pathogens including Salmonella, an efficient protection against various pathogenic bacteria could be achieved by stimulating the gut microflora to produce SCFA (Niba *et al.*, 2009). The degree of weight gains seen during the first 2 weeks before the Salmonella challenge in the groups fed with β -glucan (the BG group and the BG-X group) was lower as compared with the groups provided with normal feed (the NF group and the Neg. group). With the addition of β -glucan, however, the degree of weight gain after the Salmonella challenge was higher as compared with the NF group. This indicates that the addition of β -glucan could affect feed preference and then slightly reduce feed intake of weaned pigs at the beginning. With the addition of β -glucan, however, ADWG was significantly increased upon Salmonella infection. Previous studies have also shown that β -glucan could enhance disease resistance and help increase energy gains which led to an increase in the muscle development and production of the desired weight (Eicher *et al.*, 2006; Li *et al.*, 2006; Price *et al.*, 2010).

In the current study, the addition of β -glucan tended to increase the degree of the expression of IFN- α and IFN- β as compared with the NF group. But, this difference reached no statistical significance (Fig. 4). It has also been shown that β -glucan could boost the immune system by interacting with macrophage receptors (Chen *et al.*, 2010) or by increasing cytokine production (Lee *et al.*, 2001). β -glucan purified from *Saccharomyces cerevisiae* has been reported to enhance the production of IFN- γ and nitro oxide in pigs (Jung *et al.*, 2004; Xiao *et al.*, 2004). Pathological evaluation also showed that the gross lesions were decreased following the addition of β -glucan in the pigs (congestion, lymph node enlargement and the thickness of intestinal wall) caused by Salmonella infection as compared with the groups fed with normal feed although there was no significant difference in the microscopic lesion (microscopic congestion, inflammation and villous atrophy) scores between the groups (Fig. 5). In the current study, researchers also analyzed the combined effect of *A. pullans*-derived β -glucan with Genkwadaphnin. Genkwadaphnin has been shown to have antimicrobial effects on bacteria such as *Bacillus lentus* and *E. coli* (Cottigli *et al.*, 2001). But its effects combined with those of β -glucan would be of no significance. This is because the results demonstrated that the degree of protective effects against Salmonella infection was not significantly higher in the BG-X group as compared with the BG group.

CONCLUSION

With the dietary addition of β -glucan derived from *A. pullan*, the overall performance of weaned pigs was enhanced upon challenge with Salmonella by increasing weight gains and reducing both fecal shedding of Salmonella and the incidence of diarrhea.

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