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Purification and Anti-pathogenic Properties of Immunoglobulin Concentrates from Porcine Blood

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Abstract

During slaughtering, animal blood is typically discarded, resulting in water pollution. However, this discarded blood has valuable components, such as immunoglobulin (Ig). Although several studies have been conducted to develop methods for effective recycling of slaughterhouse blood, they have not been commercially utilized in Korea. Here, we extracted an Ig-rich fraction from porcine blood that was then subjected to various *in vitro* tests, including pathogen growth inhibition, antigenic cross-reactivity, and anti-toxin activity. The porcine immunoglobulin concentrate (PIC) was effectively purified by eliminating other components, such as albumin, and consisted of approximately 63.2±2.9% IgG and 7.2±0.4% IgM on a protein basis. The results showed that it significantly suppressed the growth of pathogenic bacteria, and bound to all tested pathogens, including both gram-positive and gram-negative species, although the degree of activity differed according to strain. The PIC bound to two types of lipopolysaccharide (LPS) obtained from *Escherichia coli* O111:B4 and *Salmonella enterica* serotype typhimurium in a concentration-dependent manner. In addition, the PIC restored the proliferation activity of the lymphoblast K-562 cells when co-incubated with pathogenic LPS. These results confirm that the PIC prepared in this study is a potentially valuable functional food material or diet supplement as an alternative to antibiotics that can protect animals from pathogenic bacteria.

Keywords slaughterhouse blood, porcine immunoglobulin concentrate, pathogen growth inhibition, antigenic cross-reactivity, anti-toxin activity

Introduction

In Korea, most slaughterhouse blood is discarded (Kim *et al.*, 2012; Yun *et al.*, 1998). Not only are the costs of slaughterhouse blood disposal high but also the disposed blood is an environmental problem since it is a major source of water pollution (Jang *et al.*, 2011; Jeon *et al.*, 2013). However, slaughterhouse blood has considerable nutritional value (Quigley *et al.*, 2004), and spray-dried animal plasma (SDAP) has been shown to improve the feed intake, weight gain, and morbidity of livestock (Pérez-Bosque *et al.*, 2016; Torrallardona, 2010). These effects are greater in animals during challenge with a pathogen or bacterial toxin (Coffey and Cromwell, 1995; Pérez-Bosque *et al.*, 2008). Furthermore, animal plasma contains useful proteins, such as fibrinogen, albumin, and globulin (Hyun and Shin, 1998;

Pérez-Bosque *et al.*, 2016; Pierce *et al.*, 2005), and the beneficial effects of the animal plasma are mainly modulated by its immunoglobulin (Ig) components (Lallès *et al.*, 2009).

Ig derived from animal plasma shows antibacterial activity against some pathogens by inhibiting their growth and anti-toxin activity through binding to lipopolysaccharide (LPS) or toxin (Han *et al.*, 2009). It was also reported that Ig could improve the growth of beneficial bacteria, such as lactic acid bacteria, and inhibit the growth of harmful pathogenic bacteria in the gut (Balan *et al.*, 2011). Although several studies that used whole blood or enzyme-treated plasma, have demonstrated effective utilization of slaughterhouse blood in Korea (Hyun and Shin, 1999; Ma *et al.*, 2001; Park, 1997), the blood has not been commercially utilized, and almost all of it is discarded.

In general, slaughterhouse blood accounts for approximately 3.5% of livestock weight, equaling approximately 7.5 L for a cow, 3.5 L for a pig, 50 mL for a duck, and 35 mL for a chicken (Jeon, 2015). Currently, approximately 523 tons of animal blood is being produced in slaughterhouses every day, and the amount of slaughterhouse blood discarded each year is about 126,000 tons (Kim, 2014). The amount of slaughterhouse blood has been consistently increasing annually, and more porcine blood is discarded than any other animal blood (Jeon, 2015). The estimated cost for the treatment of about 35,000 tons of porcine blood waste produced in 2014 was 227 million won (Kim *et al.*, 2016).

Therefore, the aim of this study was to effectively purify an Ig-rich fraction from porcine blood and evaluate its commercial potential by assessing its anti-pathogenic properties using various *in vitro* assays.

Materials and Methods

Preparation of porcine immunoglobulin concentrate (PIC)

Fresh porcine blood was collected from a local slaughterhouse, and immediately mixed with sodium citrate (1%). Plasma was separated from the porcine blood by centrifugation at 10,000×g for 20 min at 4°C (1736R; LaboGene, Korea). Calcium chloride (0.5%) was added to the plasma, which was stirred for 5 min and then incubated for 1 h at room temperature to promote serum separation. Ammonium sulfate (1.7 M) was slowly added to the serum, stirred overnight at 4°C, and then centrifuged at 10,000×g for 20 min. The precipitate was dissolved in distilled water,

and the pH was adjusted to 9. The solution was filtered through a 10-kDa cutoff membrane using ultrafiltration (Vivaflow 50 System; Sartorius, Germany), and then the pH was adjusted to 7.4. The final product (PIC) was spray-dried using a pilot spray dryer (Yoojin, Korea).

Bacterial strains and cell culture conditions

The bacterial strains used in this study were obtained from the Korean Collection for Type Cultures (Korea) and the Korean Culture Center of Microorganisms (Korea). The pathogenic bacteria were grown in brain heart infusion medium, columbia broth, marine broth, trypticase soy broth, or reinforced clostridial medium for 18 h at 37°C (Table 1). Prior to the experiments, bacteria were subcultured at least three times. For long-term storage, stock cultures were stored in fresh broth containing 20% glycerol at -80°C. The lymphoblast K-562 cell line was purchased from the Korean Cell Line Bank (Korea). These cells were cultured in Roswell Park Memorial Institute 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a humidified atmosphere of 5% CO₂.

Table 1. Pathogenic strains used in this study

No.	Bacterium	Medium
Gram positive		
1	<i>Clostridium perfringens</i> ATCC 13124	RCM
2	<i>Clostridium termitidis</i> ATCC 51846	RCM
3	<i>Eubacterium nodatum</i> ATCC 33099	TSB
4	<i>Staphylococcus aureus</i> ATCC 25923	TSB
5	<i>Staphylococcus epidermidis</i> ATCC 12228	TSB
6	<i>Streptococcus mitis</i> KCTC 5650	BHI
7	<i>Streptococcus mutans</i> ATCC 25175	BHI
8	<i>Streptococcus sobrinus</i> ATCC 33478	BHI
Gram negative		
9	<i>Actinomyces viscosus</i> ATCC 15988	BHI
10	<i>Eikenella corrodens</i> ATCC 23834	CB
11	<i>Escherichia coli</i> ATCC 9637	TSB
12	<i>Escherichia coli</i> ATCC 43896	TSB
13	<i>Fusobacterium nucleatum</i> ATCC 25586	RCM
14	<i>Prevotella intermedia</i> ATCC 49046	TSB
15	<i>Prevotella nigrescens</i> ATCC 33563	TSB
16	<i>Salmonella enterica</i> KCTC 11862	TSB
17	<i>Salmonella typhimurium</i> KCTC 40253	TSB
18	<i>Shigella flexneri</i> ATCC 12022	TSB
19	<i>Shigella sonnei</i> ATCC 9290	TSB
20	<i>Vibrio vulnificus</i> ATCC 27562	MB
21	<i>Vibrio parahaemolyticus</i> ATCC 33844	MB

ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; RCM, reinforced clostridial medium; TSB, trypticase soy broth; BHI, brain heart infusion medium; CB, columbia broth; MB, marine broth.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein composition of the purified PIC was analyzed by SDS-PAGE in a 12.5% acrylamide gel as described by Laemmli (1970). The samples, which included porcine IgG (Sigma), porcine plasma, PIC, and bovine serum albumin (BSA, Sigma), were mixed with sample buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, and 2% β -mercaptoethanol, pH 6.8) and heated for 15 min at 98°C. Electrophoresis was conducted at 20 mA (per gel) for 1 h using a Mini-Protean® Tetra System and PowerPac™ HV (Bio-Rad, USA), and the gel was stained with Coomassie® brilliant blue G-250 (Bio-Rad) for 2 h. The bands on the gel were analyzed with a Molecular Imager® GelDoc™ XR plus Imaging system and Image Lab™ software (version 5.1; Bio-Rad).

Measurement of immunoglobulins

The concentrations of IgG and IgM in the purified PIC were measured by enzyme-linked immunosorbent assay (ELISA). All reagents were purchased from Bethyl Laboratories, Inc. (USA). The sample and reference were diluted to an appropriate concentration with sample diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05 Tween 20, pH 8.0). Flat-bottom 96-well plates (Nunc) were coated with affinity-purified antibody (diluted in coating buffer), incubated for 1 h at room temperature, and then washed four times with washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). The remaining binding sites were blocked with blocking solution (1% BSA in 50 mM Tris, 0.14 M NaCl, pH 8.0) for 30 min, and then the plate was washed four times. The diluted sample and standard were added to the plate, which was incubated for 1 h, and then washed four times. An HRP detection antibody was added to each well and incubated for 1 h. The plate was washed four times, and then incubated with TMB substrate solution in the dark for 10 min. The reactions were stopped by the addition of 0.18 M H₂SO₄, and the absorbance was measured at 450 nm using a microplate reader (Emax; Molecular Devices, USA).

Pathogen growth inhibition

The PIC solution was diluted to 5% (w/v) or 10% (w/v) in buffered peptone water (BPW; Difco), and a BSA solution (10%, diluted with BPW) was prepared for use as a positive control. All of the solutions were filtered through disposable membrane filter units (0.8 and 0.45 μ m; Advantec®) before use in the growth inhibition test. Pathogen

cultures (0.1 mL) were diluted to 1×10^4 CFU/mL, and then BSA or PIC solution (0.9 mL) was added. The mixtures were cultured for 3 h at 37°C, and then incubated on optimal medium agar for 48 h at 37°C for counting. As a negative control, BPW (0.9 mL) without any protein was used. All measurements were performed in triplicate, and the percent growth was calculated as follows: Growth (%) = $(\text{Log}_{10}N_m / \text{Log}_{10}N_c) \times 100$; where N_c and N_m are the numbers of pathogens in the control and in test mixture, respectively, after 3 h incubation.

Antigenic cross-reactivity

The antigenic cross-reactivity of the PIC was investigated using the method described by Tomita *et al.* (1995), with a slight modification. Briefly, the pathogen cultures were incubated for 18 h at 37°C and then washed three times with phosphate buffered saline (PBS; Sigma), and the optical density (OD) at 610 nm of the final pathogen suspension was adjusted to 1.0. The pathogenic antigens were bound to a flat-bottom 96-well plate by incubation overnight at 4°C, and washed four times with washing buffer (PBS containing 0.05% Tween 20). The remaining binding sites were blocked by incubation with blocking buffer (3% BSA in PBS) for 1 h at 37°C and then washed four times. Then, the PIC solution (100 μ L of 10 mg/mL) was added to each well, and the plate was incubated for 2 h at 37°C. After the plate was washed four times, a HRP-conjugated goat anti-pig IgG antibody (Bethyl) was added to each well and incubated for 2 h at 37°C. The absorbance was measured as described above. The positive control was PIC solution without any pathogen suspension. All measurements were performed in triplicate, and the percentage of antigenic cross-reactivity was determined according to the following formula:

$$\text{Antigenic cross-reactivity (\%)} = (\text{mean OD}_{450} \text{ of pathogenic antigen} / \text{mean OD}_{450} \text{ of positive control}) \times 100$$

Anti-toxin activity

The anti-toxin activity of the PIC against LPS was determined using the method of Yu and Kanost (2002), with a slight modification. Flat-bottom 96-well plates were coated with two types of LPS from *E. coli* O111:B4 or *S. enterica* serotype Typhimurium (10 μ g or 0.1 μ g; Sigma) by overnight incubation at 4°C, and then washed four times with washing buffer (PBS containing 0.05% Tween 20). After washing, the plate was blocked by incubation with blocking buffer (3% BSA in PBS) for 1 h at 37°C, and

then washed again four times. PIC diluted in PBS was added to each well at various concentrations and incubated for 2 h at 37°C. After the plate was washed four times, an HRP-conjugated goat anti-pig IgG antibody (Bethyl) was added to each well, and the plate was incubated for 2 h at 37°C. The absorbance was measured as described above.

In addition, the binding activity of the PIC to LPS was investigated using the lymphoblast K-562 cell line and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the method described by Han *et al.* (2009), with a slight modification. K-562 cells (1×10^4 cells/well) were cultured in a 96-well plate (Nunc) for 24 h at 37°C in the presence of 5% CO₂. PIC solution (50 µL) prepared at various concentrations was mixed with 50 µL of LPS solution (160 µg/mL) from *E. coli* O111:B4 or *S. enterica* serotype typhimurium, and the mixture was incubated for 30 min at 37°C. The mixtures were transferred to the plate, which was incubated for 72 h at 37°C. Then, 100 µL of MTT solution (1 mg/mL) was added to each well, and the plate was incubated for 4 h at 37°C. The medium was removed, and 150 µL of dimethyl sulfoxide was added. The plate was shaken for 10 min at room temperature, and then the absorbance at 540 nm was measured using a microplate reader.

Statistical analysis

The results were expressed as mean \pm SEM, and data were analyzed using the General Linear Models procedure and the PROC TTEST in the SAS program (SAS version 9.2; SAS Institute Inc., USA). Statistical significance was accepted at $p < 0.05$.

Results

Composition of PIC

The Ig concentrates from porcine blood were effectively purified by eliminating other components, such as albumin (Fig. 1). The heavy and light chains of Ig migrated at approximately 49 and 26 kDa on the SDS-PAGE gel, respectively. The ELISA results showed that, on a protein basis, the PIC consisted of approximately $63.2 \pm 2.9\%$ IgG and $7.2 \pm 0.4\%$ IgM.

Pathogen growth inhibition

The PIC significantly suppressed the growth of all tested pathogenic bacteria when compared to their growth in the control or BSA treatment. However, the degree of growth suppression activity differed according to pathogen strain

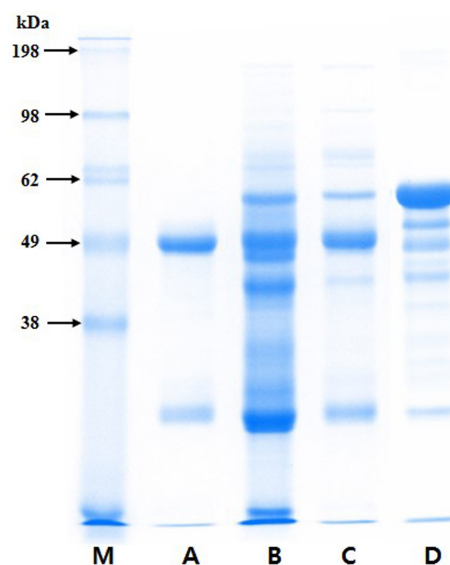


Fig. 1. SDS-PAGE of immunoglobulin concentrate purified from porcine blood. M, protein marker; A, porcine IgG; B, porcine plasma; C, porcine immunoglobulin concentrate purified in this study; D, bovine serum albumin.

and PIC concentration (5% or 10%). Representative inhibitory activities of the PIC against the growth of various pathogens are shown in Fig. 2. When treated with 10% PIC, *A. viscosus* ATCC 15988 and *S. mutans* ATCC 25175 were the most effectively suppressed, by about 45-55%. In contrast, the BSA solution did not exhibit anti-growth activity against the tested pathogens.

Antigenic cross-reactivity

As shown in Fig. 3, the antigenic cross-reactivity of the PIC varied according to the pathogen strain. The PIC was able to bind to all the tested pathogens, including both gram-positive and gram-negative bacteria, shown in Table 1. In particular, the binding activity of PIC to some pathogens, such as *C. perfringens* ATCC 13124, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *E. corrodens* ATCC 23834, *E. coli* ATCC 9637, and *V. vulnificus* ATCC 27562 was very close to that of the control.

Anti-toxin activity

The binding of PIC to LPS from *E. coli* O111:B4 and *S. enterica* serotype typhimurium was PIC concentration-dependent (Fig. 4). The slope of the curves rapidly increased to about 50% saturation and then increased moderately with a corresponding increase in PIC concentration. When treated with a larger amount of LPS (10 µg/well), the absorbance values were higher than samples treated

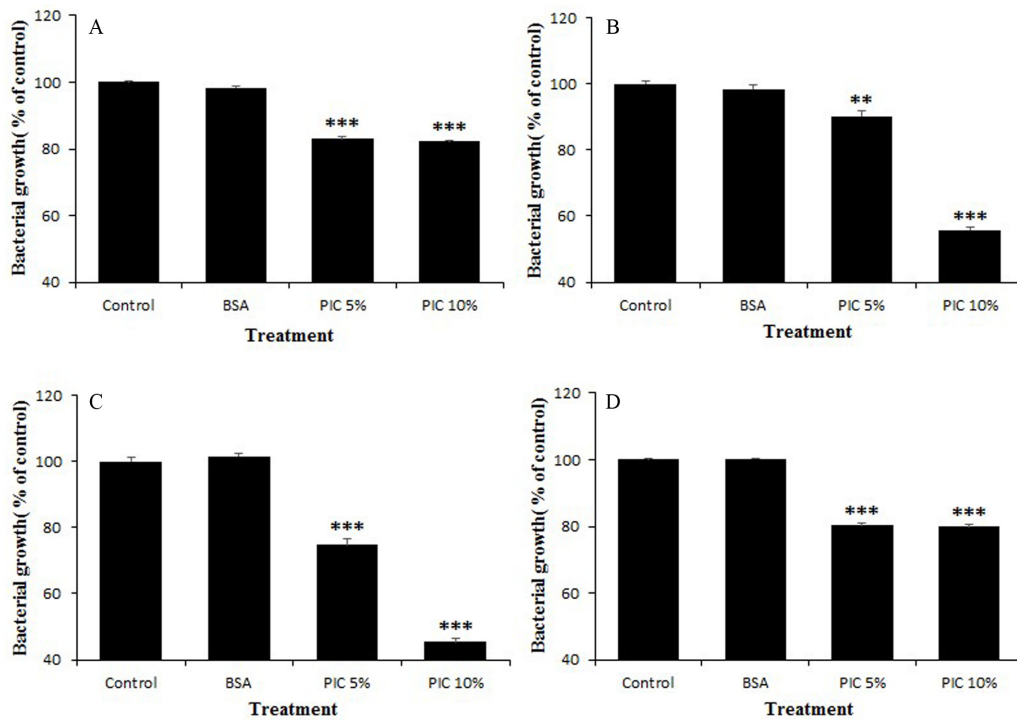


Fig. 2. Inhibition of pathogen growth by porcine immunoglobulin concentrate. Values are expressed as means \pm SEM. Differences were considered to be statistically significant at $p < 0.01$ (**) or $p < 0.001$ (***). BSA, bovine serum albumin; PIC, porcine immunoglobulin concentrate. A, *Clostridium perfringens* ATCC 13124; B, *Streptococcus mutans* ATCC 25175; C, *Actinomyces viscosus* ATCC 15988; D, *Escherichia coli* ATCC 9637.

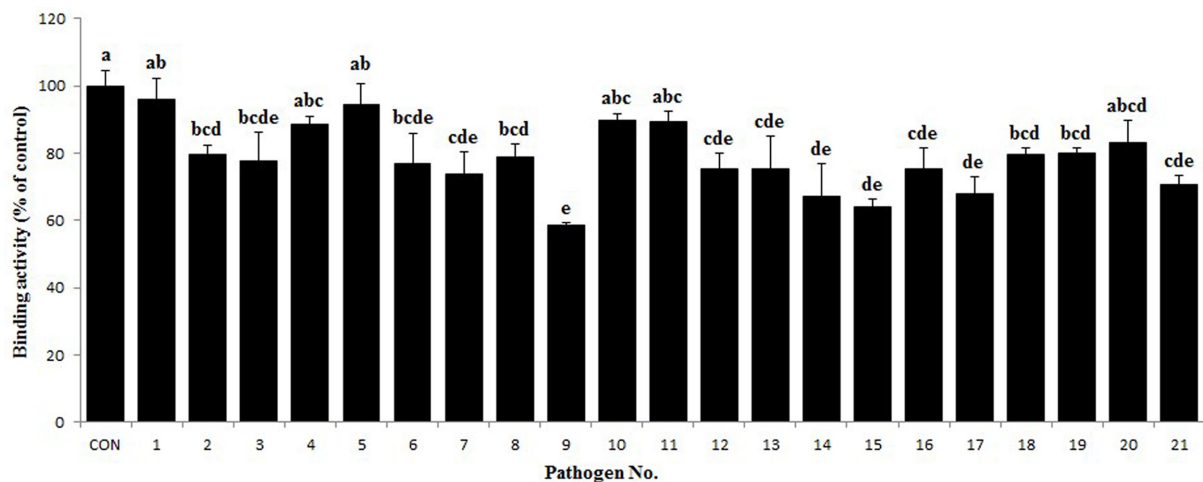


Fig. 3. Antigenic cross-reactivity of porcine immunoglobulin concentrate against various pathogens. Values are expressed as means \pm SEM. Means without a common letter differ significantly ($p < 0.001$). The pathogens used are listed in Table 1. CON, control.

with a smaller amount of LPS concentration (0.1 $\mu\text{g}/\text{well}$). The anti-toxin activity of the PIC was also examined using the K-562 cell line. The growth rate of the K-562 cells was decreased by treatment with LPS (160 $\mu\text{g}/\text{mL}$) but, this reduction was restored by the addition of PIC (Fig. 5).

Treatment with PIC at 300-500 $\mu\text{g}/\text{mL}$ markedly increased the viability of the K-562 cells ($p < 0.05$ or $p < 0.01$), which means that the PIC could bind to LPS, reducing the pathogenicity of LPS.

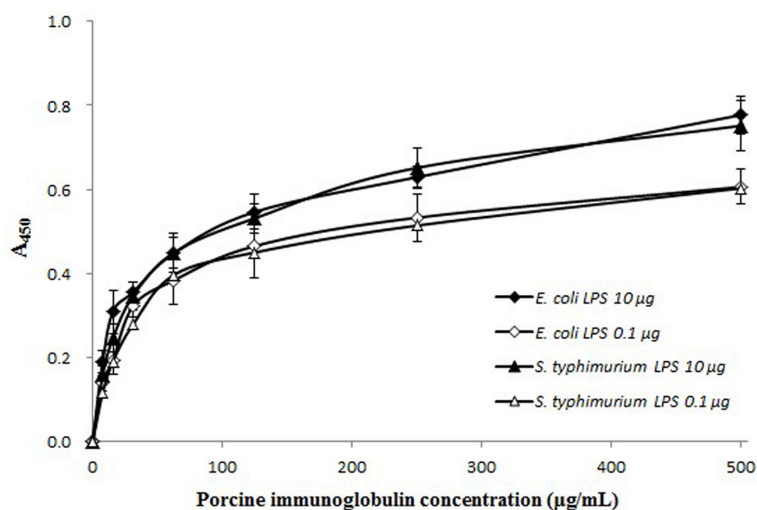


Fig. 4. The binding of porcine immunoglobulin concentrate to two types of lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 and *Salmonella enterica* serotype Typhimurium. Values are expressed as means±SEM. A_{540r}, Absorbance at 540 nm.

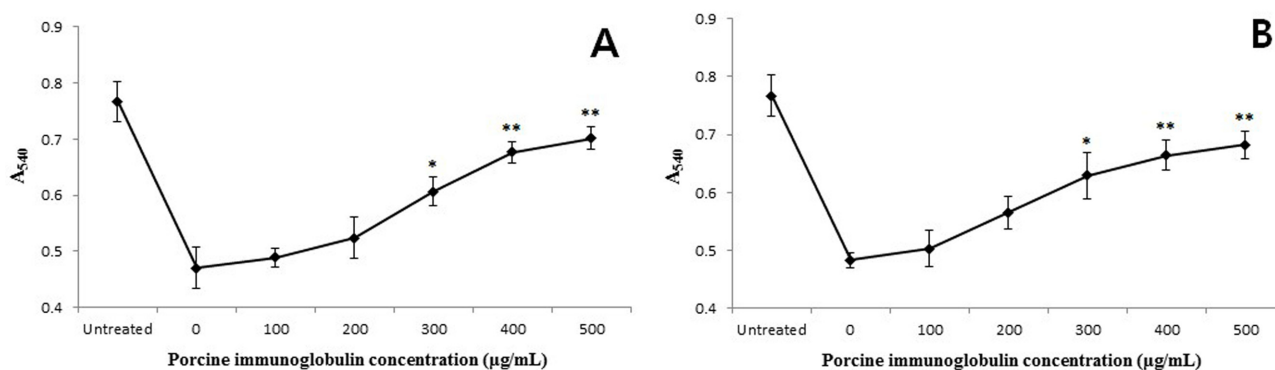


Fig. 5. Effect of porcine immunoglobulin concentrate on the proliferation of lymphoblast K-562 cells treated with lipopolysaccharide (LPS). Values are expressed as means ± SEM. Asterisks indicate significant differences, (*) $p < 0.05$ and (**) $p < 0.01$. A, *Escherichia coli* O111:B4 LPS (160 µg/mL); B, *Salmonella enterica* serotype Typhimurium LPS (160 µg/mL). A_{540r}, Absorbance at 540 nm.

Discussion

It is known that administration of animal plasma in livestock improves intestinal barrier function and mucosal inflammation as well as growth performance (Bosi *et al.*, 2004; Peace *et al.*, 2011). Pierce *et al.* (2005) reported that the beneficial effects of animal plasma were primarily mediated by the Ig fraction. Administration of Ig increased the levels of anti-inflammatory cytokines in the intestinal mucosa (Pérez-Bosque *et al.*, 2008) and reduced the expression of pro-inflammatory cytokines, such as tumor necrosis factor alpha, interferon gamma, interleukin 2, and interleukin 17, in various *in vitro* models (Bosi *et al.*, 2004; Moretó and Pérez-Bosque, 2009). Other studies have shown that dietary supplements with Ig imp-

roved intestinal health and immunity in clinical trials (Asmuth *et al.*, 2013; Wilson *et al.*, 2013). The Ig concentrate isolated from porcine blood in this study contained approximately 63% IgG on a protein basis. This suggests its potential value as a supplement for functional food or animal diet, because commercial Ig fractions generally contain less than 50% IgG.

In the present study, the PIC not only inhibited the growth of various pathogens but also directly bound to their surface. It was reported that IgM had antibacterial activity and decreased the production of matrix metalloproteinase-1 caused by LPS (Zhou *et al.*, 2007). Petschow *et al.* (2014) reported that Ig, especially IgG, had antibacterial activity against pathogenic bacteria, because Ig inhibited the ability of pathogens or their toxins, which mig-

rated through the mucus layer into epithelial cells, and damaged pathogens through direct binding. Furthermore, the Fab fragment of IgG imparts diversity, and the Fc fragment can interact with the Fc gamma receptors on some immune cells, including macrophages and monocytes, thus stimulating their phagocytic activity (Petschow *et al.*, 2014). Several studies have reported that the Ig present in SDAP reduced pathogen adhesion to the gut mucosa as well as the intestinal inflammation caused by pathogenic bacteria, thus providing immunological benefits (Pérez-Bosque and Moretó, 2010; Van Dijkstra *et al.*, 2001).

PIC can bind to LPS on the surface of gram-negative bacteria and reduce its growth-repressing action on K-562 cells. Hence, PIC may protect the intestine from diseases caused by gram-negative bacteria and their toxins. Bacterial LPS can cause various tissue pathologies and intestinal permeability disorders, such as Crohn's disease, necrotizing enterocolitis, and inflammatory bowel disease, by increasing intestinal epithelial tight junction permeability (Guo *et al.*, 2013; Magata *et al.*, 2015). The enterotoxigenic *E. coli* (ETEC) and *Salmonella* strains used in the present study induce severe enteropathy. ETEC adheres to the intestinal epithelium through various routes using colonization factors, which include more than 20 known multimeric structures, and it is the most common cause of diarrhea (O'Ryan *et al.*, 2015). *Salmonella* strains, as food-borne enteropathogens, cause serious gastrointestinal invasive diseases (MacLennan *et al.*, 2014). The Ig present in plasma might provide effective protection from these bacterial toxin-involved intestinal diseases, and increase phagocytic activity and lymphocyte proliferation (Balan and Moughan, 2013; Niewold *et al.*, 2007). Previous studies have also reported that supplementation with SDAP reduced the expression of inflammatory cytokines and prevented the inflammatory diseases caused by LPS (Pérez-Bosque *et al.*, 2016; Touchette *et al.*, 2002).

Conclusion

In this study, we effectively isolated Ig concentrate from porcine blood. We demonstrated that the produced PIC has anti-bacterial and anti-toxin activities, which may provide protection against pathogenic bacteria. The commercial development of PIC would not only reduce the cost burden of slaughterhouse blood disposal but may also be a potentially valuable functional food material or supplement as an alternative to antibiotics in livestock diets.

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