Effects of intranasal administration with *Bacillus subtilis* on immune cells in the nasal mucosa and tonsils of piglets

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Received August 17, 2017; Accepted December 15, 2017

DOI: 10.3892/etm.2018.6093

Abstract. The nasal mucosa is the body's first barrier against pathogens entering through the respiratory tract. The respiratory immune system of pigs has more similarities with humans than the mouse respiratory system does, and so was selected as the animal model in the present study. To evaluate the effects of *Bacillus subtilis* as a potential probiotic to stimulate local immune responses, piglets were intranasally administered with Dylight 488-labeled *B. subtilis* (WB800-green fluorescent protein). The results revealed that *B. subtilis* was able to reach the lamina propria of the nasal mucosa, nasopharyngeal tonsils and soft palate tonsils. Piglets were subsequently administered intranasally with *B. subtilis* (WB800) at 3, 12 and 28 days. The results revealed that, following administration with *B. subtilis*, the number of dendritic cells, immunoglobulin A^+ B cells and T cells in the nasal mucosa and tonsils significantly increased ($P<0.05$). No obvious differences were observed in the morphological structure following *B. subtilis* administration. There were no statistical differences were observed in the expression of interleukin (IL)-1β, tumor necrosis factor-α and IL-8 mRNA between the *B. subtilis* treated group and the control group in the nasal mucosa, nasopharyngeal tonsil or soft palate tonsil. Toll-like receptor (TLR)-2 and TLR-9 mRNA expression in the tonsils was significantly increased following *B. subtilis* administration compared with the control group ($P<0.05$). The results demonstrate that *B. subtilis* administration increases the number of immune cells in the nasal mucosa and tonsils of piglets and stimulates nasal mucosal and tonsillar immunity. The present study lays the foundation for further study into the intranasal administration of *B. subtilis* in humans to enhance the immunity of human nasal mucosa to respiratory diseases.

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Key words: piglets, intranasal administration, nasal mucosa, tonsil, *Bacillus subtilis*, immune cells

Introduction

Infectious respiratory diseases, including mycoplasma pneumonia and influenza, present a serious threat to public health (1,2). The nasal mucosa is the primary invasion site for a number of pathogenic microorganisms and nasal immunization has the potential to increase mucosal immunity (3). The anatomy of the porcine respiratory system is similar to that of humans (more so than mice) and is considered to be a good animal model (4,5). The tonsils are located near the nasal cavity, which is understood to serve the same role as the Waldeyer's ring in humans (6). Waldeyer's ring is located at the entrance to the digestive and respiratory tracts where it acts as a key component in the mucosal-associated lymphoid system (7). B and T cells are located within the nasal-associated lymphoid tissue and tonsils with antigen-presenting cells (APCs), including dendritic cells (DCs), interspersed among them (8,9). The nasal mucosa and tonsils are considered to be a potential target for nasal vaccines as they serve an important role in antigen recognition and immune activation following intranasal administration, which is similar to gut-associated lymphoid tissue in the gastrointestinal tract (10,11).

There are high numbers of DCs distributed under the nasal epithelium and, as the most ubiquitous professional APCs in the nasal mucosa and tonsils, they serve an important role in immune surveillance (12,13). DCs are able to present antigens and activate T cell responses (14,15).

Probiotics are defined as live microorganisms that confer a health benefit when administered at adequate concentrations (16). It has previously been suggested that probiotic bacteria interact with epithelial and immune cells from the innate immune system to reinforce the mucosal barrier (17). Probiotic bacteria may be sampled by DCs as whole cells or their antigenic fragments (17). *Bacillus subtilis* is a common probiotic with good biological safety that is able to effectively antagonize pathogenic bacteria (18). It has been reported that *B. subtilis* effectively enhances the innate immunity of the intestinal tract; however, whether *B. subtilis* is able to enhance the innate immunity of the nasal mucosa and tonsils remains unknown (19). The spores of *B. subtilis* have been successfully used for antigen delivery and they may also be used as a mucosal adjuvant for the H5N1 vaccine, in which they significantly enhance the effect of intranasal immunization (20,21). The mechanism by which *B. subtilis* spores act as a potential

immune adjuvant and whether this effect is associated with immune cells in the nasal mucosa and tonsils require further investigation.

In the present study, pigs were selected as the animal model to investigate the effects of *B. subtilis* on immune cells in the nasal mucosa and tonsils. Changes to the immune cells in the nasal mucosa and tonsils following intranasal administration with *B. subtilis* were observed. The results of the present study may be valuable for further study into an intranasal vaccines with *B. subtilis* as a mucosal adjuvant.

Materials and methods

Animals and reagents. A total of 6 (age, 1-month-old; weight, 13-16 kg) and 8 (age, 1-day-old; weight, 1.10-1.30 kg) male cross-bred Duroc/Landrace/Yorkshire piglets were obtained from Jiangsu Huai'an Pig Farm (Huai'an, China). A total of 2 cross-bred Landrace/Yorkshire sows (age, 1 year and 6 months; weight, 140-145 kg; Jiangsu Huai'an Pig Farm) were used to nurse the piglets. The pigs and piglets were housed in Jiangsu Huai'an Pig Farm with a constant humidity (60%) and temperature (26˚C) at 12 h light/dark cycle with free access to water and food. *B. subtilis* WB800 was obtained from the College of Plant Protection Nanjing Agricultural University (Nanjing, China).

Intranasal administration. A total of 6 1-month-old male piglets were randomly equally divided into two groups (n=3) as follows: The control group was intranasally administrated with PBS (500 µl/nostril) and the *B. subtilis* group was intranasally administrated with Dylight 488 (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA)-labeled *B. subtilis* WB800 spores (10^8 cfu/nostril). The procedure was repeated after 1 h. Piglets were euthanized by intravenous injection of sodium pentobarbital (100 mg/kg) 2 h later as previously described (22). All procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) and followed the National Institutes of Health guidelines for the performance of animal experiments. Following sacrifice, the pigs were decapitated, the lower jaw and skin were removed and the muscles around the nasal cavity were removed. The nasal cavities, nasopharyngeal tonsils and soft palate tonsils were removed, fixed in 4% paraformaldehyde at room temperature for 6 h, embedded in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and cut into 8-µm-thick sections for immunofluorescence staining.

A total of 8 1-day-old male piglets were bred in a pathogen free environment with the aforementioned conditions and randomly divided into two groups (n=4): The control group and the *B. subtilis* group. While the piglets were suckling, sows had free access to water and feed (containing no antibiotics). No pigs succumbed during the experiment. The *B. subtilis* group was intranasally administrated with 200 µl/nostril *B. subtilis* WB800 (10^{10} cfu/ml) on day 3, 500 µl/nostril on day 12 and 1 ml/nostril on day 21; these doses were selected from previous studies (22-26). The control group was treated with the same volumes of PBS. On day 28 the piglets were euthanized by intravenous injection of sodium pentobarbital. The nasal cavities, nasopharyngeal tonsils and soft palate tonsils were extracted and fixed in Bouin's fluid (Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing, China) for 48 h at 20-25˚C for histological analysis, or stored at -80˚C for the detection of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-8, IL-6, porcine β-defensin 2 (pBD-2), toll-like receptor (TLR)-2, TLR-9 mRNA using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The nasal cavities were subsequently decalcified by dipping them in formic acid decalcifying solution (Nanjing SenBeiJia Biological Technology, Co., Ltd.) for 1 week. The tissues were embedded in paraffin, cut into 5-µm-thick sections, dewaxed in xylene, rehydrated in a graded series of ethanol (100, 90, 80 and 70%, each for 1 min) and washed in PBS for immunohistochemistry, immunofluorescence, and hematoxylin and eosin (H&E) staining. The sections were immersed in hematoxylin for 30 sec, PBS for 5 min and eosin for 5 sec. The sections were dehydrated through increasing concentrations of ethanol (70, 80, 90 and 100%, each for 1 min) and xylene. All processes are performed at room temperature.

Immunohistochemistry. The paraffin embedded sections were dewaxed in xylene and rehydrated in a graded series of ethanol (100, 90, 80 and 70%, each for 1 min). Sections were subsequently poached in a citrate buffer (pH 6) at 90-95˚C for 15 min to retrieve antigens. The sections were treated with 0.3% hydrogen peroxide at room temperature for 15 min to quench endogenous peroxidase. The sections were blocked with 5% normal goat serum, 5% bovine serum albumin (BSA) and 5% normal rabbit serum (all Boster Biological Technology, Pleasanton, CA, USA) for 30 min at room temperature. The sections were subsequently incubated with primary antibodies 1-4 listed in Table I for 12 h at 4˚C. Biotinylated secondary antibodies 1-4 listed in Table I were added to the sections for 1 h at room temperature. Positive cells were visualized by treatment with diaminobenzidine (DAB) for 60 min at room temperature, the sections were then sealed with neutral balata. The negative control was treated in the same way except the primary antibodies were omitted. The sections were visualized using a light microscope (Olympus CX23; Olympus Corporation, Tokyo, Japan) at a magnification of x400. A total of 15 fields of each tissue from each piglet were assessed for the statistical analysis.

Immunofluorescence and confocal microscopy. The frozen sections were incubated with 5% BSA for 20 min at room temperature and subsequently incubated with PE-conjugated goat anti-pig CD11b primary antibodies (Table I) at 4˚C for 12 h. The sections were washed in PBS and incubated with AlexaFluor 594-conjugated donkey anti-goat immunoglobulin G (IgG)2b secondary antibodies at room temperature for 1 h (Table I). The sections were subsequently stained with DAPI solution (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 5 min. The negative control was treated in the same way except the primary antibodies were omitted. The sections were visualized using a Zeiss LSM710 confocal microscope (Zeiss AG, Oberkochen, Germany) at a magnification of x1,000.

The paraffin embedded sections were rinsed and subjected to antigen retrieval as described above. The serum incubation and antibody incubations were the same as for the frozen sections described above. The primary antibodies used

Table I. Information of antibodies.

No.	Primary antibodies				Secondary antibodies			
	Name	Cat. no.	Dilution	Supplier	Name	Cat. no.	Dilution	Supplier
1	Rabbit anti-pig CD3	ab16669	1:400	Abcam, Cambridge, MA, USA	SABC-POD (rabbit IgG) kit	SA1022	1:1	Boster Biological Technology, Pleasanton, CA, USA
2	Goat anti-pig IgA	A100-102P	1:200	Bethyl Laboratories, Inc., Montgomery, TX, USA	SABC-POD (goat IgG) Kit	SA1023	1:1	Boster Biological Technology
3	Mouse anti-pig CD4	sc1140	1:200	Santa Cruz Biotechnology, Inc., Dallas, TX, USA	SABC-POD (mouse IgG) kit	SA1021	1:1	Boster Biological Technology
4	Mouse anti-pig CD8	NBP1-28237	1:200	Novus Biologicals, LLC, Littleton, CO, USA	SABC-POD (mouse IgG) kit	SA1021	1:1	Boster Biological Technology
5	PE-conjugated goat anti-pig CD11b	ab62817	1:200	Abcam, Cambridge, MA, USA	AlexaFluor 594-conjugated donkey anti-goat IgG2b	A-11058	1:200	Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA
6	FITC-conjugated mouse anti-pig MHC II	MCA2314F	1:200	Bio-Rad Laboratories, Inc., Hercules, CA, USA	AlexaFluor 488-conjugated donkey anti-mouse IgG1	A-21202	1:200	Invitrogen; Thermo Fisher Scientific, Inc.
7	Rabbit anti-human TLR-2	PA1-41045	1:1,000	Thermo Fisher Scientific, Inc., Waltham, MA, USA	HRP-conjugated goat anti-rabbit IgG	A6154	1:5,000	Sigma Aldrich; Merck KGaA, Darmstadt, Germany
8	Rabbit anti-human TLR-2	PA5-20202	1:1,000	Thermo Fisher Scientific, Inc., Waltham, MA, USA	HRP-conjugated goat anti-rabbit IgG	A6154	1:5,000	Sigma Aldrich; Merck KGaA
9	Mouse anti-pig GAPDH	AF0006	1:1,000	Beyotime Institute of Biotechnology, Haimen, China	HRP-conjugated goat anti-mouse IgG	A9917	1:5,000	Sigma Aldrich; Merck KGaA

IgG, immunoglobulin G; TLR, toll-like receptor; CD, cluster of differentiation; MHC, major histocompatibility complex; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; SABC-POD, Strept Avidin-Biotin Complex-Peroxidase.

Table II. Oligonucleotide polymerase chain reaction primers.

Gene	GenBank accession number	Primers sequence (5'-3')	Orientation
TNF-α	X57321.1	CACCACGCTCTTCTGCCTACTGC	Forward
		TCGGCTTTGACATTGGCTACAA	Reverse
IL-8	NM213867	TAGGACCAGAGCCAGGAAGA	Forward
		GAACTGCAGCCTCACAGAGA	Reverse
IL-1β	M86725.1	AAGTGATGGCTAACTACGGTGAC	Forward
		ATCTGCCTGATGCTCTTGTTCC	Reverse
IL-6	M86722.1	TGGATAAGCTGCAGTCACAG	Forward
		ATTATCCGAATGGCCCTCAG	Reverse
pBD-2	AY506573.1	ACCTGCTTACGGGTCTTG	Forward
		CTCTGCTGTGGCTTCTGG	Reverse
TLR-2	AB072190	ACATGAAGATGATGTGGGCC	Forward
		TAGGAGTCCTGCTCACTGTA	Reverse
TLR-9	AB071394	GTGGAACTGTTTTGGCATC	Forward
		CACAGCACTCTGAGCTTTGT	Reverse
β-actin	U07786.1	CATCACCATCGGCAACGA	Forward
		GCGTAGAGGTCCTTCCTGATGT	Reverse

TNF, tumor necrosis factor; IL, interleukin; TLR, toll-like receptor; pBD, porcine β-defensin 2.

were PE-conjugated goat anti-pig CD11b and fluorescein isothiocyanate-conjugated mouse anti-pig major histocompatibility complex (MHC)II antibodies (Table I). The fluorescent secondary antibodies used were AlexaFluor 594-conjugated donkey anti-goat IgG2b and AlexaFluor 488-conjugated donkey anti-mouse IgG1 (Table I). A total of 15 different fields of each tissue type were assessed from each piglet for statistical analysis by using Image Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

RNA isolation and RT-qPCR. Total RNA was extracted from mucosa segments using a TRIzol™ Plus RNA Purification kit (Thermo Fisher Scientific, Inc.). Total RNA (2 mg) reverse transcribed using PrimeScript™ RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. Reverse transcription was performed at 37˚C for 50 min and then at 70˚C for 15 min. A total of 2 μl diluted cDNA (vol/:vol, 1:20) was used for RT-qPCR analysis, which was performed with an ABI 7500 PCR system (Life Technologies; Thermo Fisher Scientific, Inc.) using SYBR-Green qPCR Master Mix (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. The thermocycling protocol was 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 31 sec. The data was normalized to the β-actin housekeeping gene to account for repeated measures. The specific primers used for PCR are listed in Table II. The porcine TNF-α, IL-8, IL-1β, IL-6, pBD-2, TLR-2 and TLR-9 primers were taken from Deng *et al* (27) and Khoufache *et al* (28). PCR products were analyzed using a melting curve and the results (fold change) were calculated using the $2^{-\Delta\Delta Cq}$ method (29).

ELISA and western blot analysis. Tissue samples (50 mg) were homogenized in PBS using a Brinkman homogenizer and subjected to three freeze-thaw cycles (-20˚C and 37˚C). The homogenates were subsequently centrifuged for 5 min at 5,000 x g at 4˚C. Levels of IL-1β, IL-8 and TNF-α proteins in the supernatant were analyzed using IL-1β (cat. no. 70-EK101B2), IL-8 (cat. no. 70-EK1082) and TNF-α (cat. no. 70-EK1822) ELISA kits [all Hangzhou MultiSciences (Lianke) Biotech, Co, Ltd., Hangzhou, China] according to the manufacturer's protocol. Each sample was run in triplicate.

Tissue samples (50 mg) were homogenized in 500 μl radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) with a Brinkman Homogenizer. Total protein was extracted using a Protein Extraction kit (Beyotime Institute of Biotechnology) and protein content was measured using a bicinchoninic acid assay according to the manufacturer's protocol (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The homogenates were subsequently centrifuged for 15 min at 14,000 x g at 4˚C. The supernatant was diluted in a 5:1 ratio with SDS-PAGE Sample Loading Buffer (5X; Beyotime Institute of Biotechnology) and heated to 98˚C for 10 min. Proteins (40 mg/lane) were separated in an 8% gel via SDS-PAGE. The protein was then transferred onto a 0.45 μm-pore polyvinylidene difluoride membrane (Immun-Blot®; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 100 V for 1 h. The membranes were blocked with 7% skim milk in PBS with 0.1% Tween-20 at room temperature for 2 h. The TLR-2 and TLR-9 proteins were detected using western blot analysis with rabbit anti-human TLR-2 and rabbit anti-human TLR-9 polyclonal antibodies (Table I) for 10 h at 4˚C, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Table I) for 1 h at room temperature. The signals were detected using Supersignal West Pico kit (Thermo Fisher Scientific, Inc.) and subjected to an ImageQuant LAS-4000 imaging system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The intensity of the bands in terms of

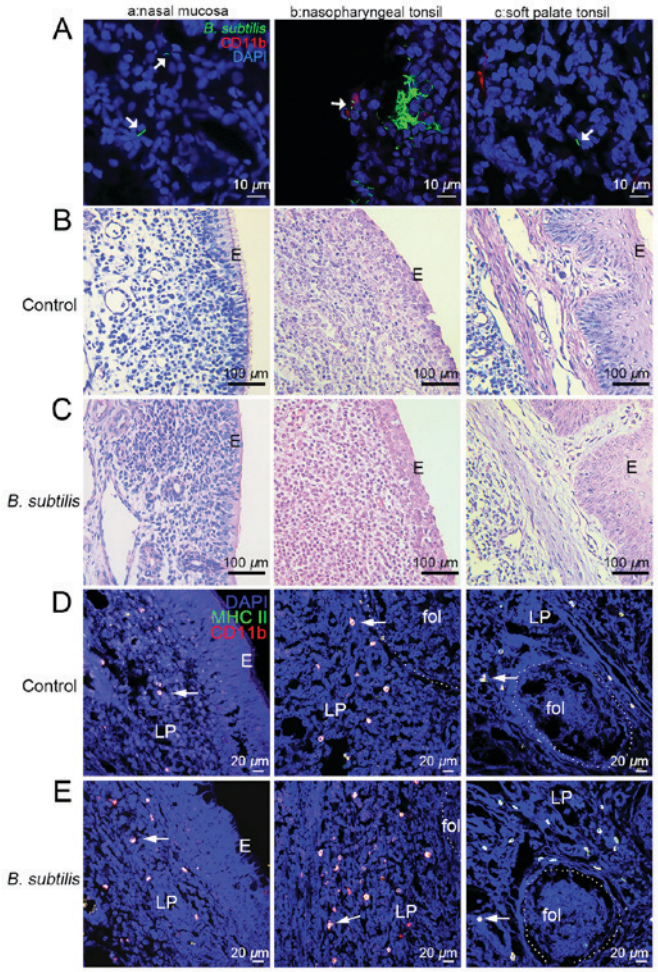

Figure 1. Effects of intranasal administration of *B. subtilis* on DCs in the nasal mucosa, nasopharyngeal tonsil and soft palate tonsils of piglets. (A) Immunofluorescent staining was performed to detect the prescence of Dylight 488-labeled *B. subtilis* (green) in the (A-a) lamina propria of nasal mucosa, (A-b) nasopharyngeal tonsil and (A-c) soft palate tonsil. $CD11b^+$ DCs were labelled as red and DAPI was labelled as blue. (n=3/group). Hematoxylin and eosin staining was performed on (B) control samples and (C) *B. subtilis* samples in the (B-a and C-a) nasal mucosa, (B-b and C-b) nasopharyngeal tonsil and (B-c and C-c) soft palate tonsil. Immunofluorescent staining was performed on (D) control samples and (E) *B. subtilis* samples. $CD11b^+$ (red) and MHC II^+ (green) DCs (arrows) were detected in the (D-a and E-a) nasal mucosa, (D-b and E-b) nasopharyngeal tonsil, (D-c and E-c) and soft palate tonsil. $CD11b^+$ MHC II^+ DCs were labelled as orange/yellow and DAPI was labelled as blue (n=4/group). E, epithelium; LP, lamina propria; fol, lymphoid follicle; DCs, dendritic cells; CD, cluster of differentiaion.

density was measured by Image Pro Plus version 6.0 (Media Cybernetics, Inc.) and normalized against GAPDH expression. Three independent experiments and appropriate gel exposures yielded very similar results for each treatment modality.

Statistical analysis. Measurements and cell counts were performed using Image Pro Plus version 6.0 (Media Cybernetics, Inc.). Results are expressed as the mean ± standard error of the mean. One-way analysis of variance, followed by Duncan's test, was employed to determine statistical differences among multiple groups and t-test was employed to determine the same between two groups. Histological scores were analyzed by the Mann-Whitney U test. $P<0.05$ indicated that the difference between groups was statistically significant.

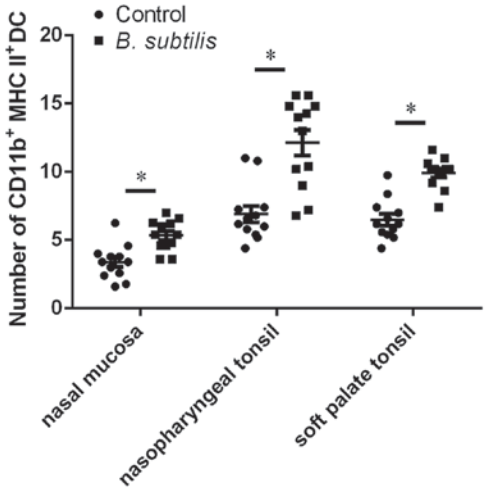

Figure 2. Point graph of the number of $CD11b^+MHC\ II^+$ DCs in the nasal mucosa, nasopharyngeal tonsil and soft palate tonsil following intranasal administration of *B. subtilis*. The data are presented as the mean ± standard error of the mean (n=4/group). The error bars indicate the standard error. $^*P<0.05$. CD, cluster of differentiation; MHC, major histocompatibility complex; DC, dendritic cells.

Results

Analysis of B. subtilis location following intranasal administration. Dylight 488-labeled *B. subtilis* were observed in the lamina propria of the nasal mucosa, nasopharyngeal tonsils and soft palate tonsils, and the concentration of *B. subtilis* was markedly higher in the nasopharyngeal tonsil compared with the nasal mucosa or the soft palate tonsils (Fig. 1A). *B. subtilis* were also observed inside the $CD11b^+$ DCs (Fig. 1A-b). The morphological structure of the tissue did not appear to change and the epithelial integrity remained intact following intranasal administration with *B. subtilis* (Fig. 1B and C), which indicates that intranasal administration with *B. subtilis* did not cause pathological changes to the tissues.

Effect of B. subtilis on DCs in the nasal mucosa and tonsils. Immunofluorescent analysis via dual staining with antibodies specific to the DC markers to detected DCs. $CD11b^+$ cells were stained red, MHC II^+ cells were stained green and double positive cells were stained bright yellow (arrows; Fig. 1D and E). There were several double positive cells and they were located in the lamina propria particularly around the lymphatic follicles (fol; dotted circles). The DCs were round or polygonal and distributed in the lamina propria particularly around the lymphatic follicles. $CD11b^+$ MHC II^+ DCs were significantly increased in the nasal mucosa, nasopharyngeal tonsil and soft palate tonsil following administration with *B. subtilis* compared with the control ($P<0.05$; Fig. 2).

Effect of B. subtilis on T cells in the nasal mucosa and tonsils. Immunohistochemical staining was performed on the paraffin embedded samples (Fig. 3). The results revealed that $CD3^+$, $CD4^+$ and $CD8^+$ T cells were widely distributed in the lamina propria of the nasal mucosa, soft palate tonsils and nasopharyngeal tonsils (Fig. 3A-F). The cells positively stained for $CD3^+$, $CD4^+$ and $CD8^+$ appeared brown. The number of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in the nasal mucosa, nasopharyngeal

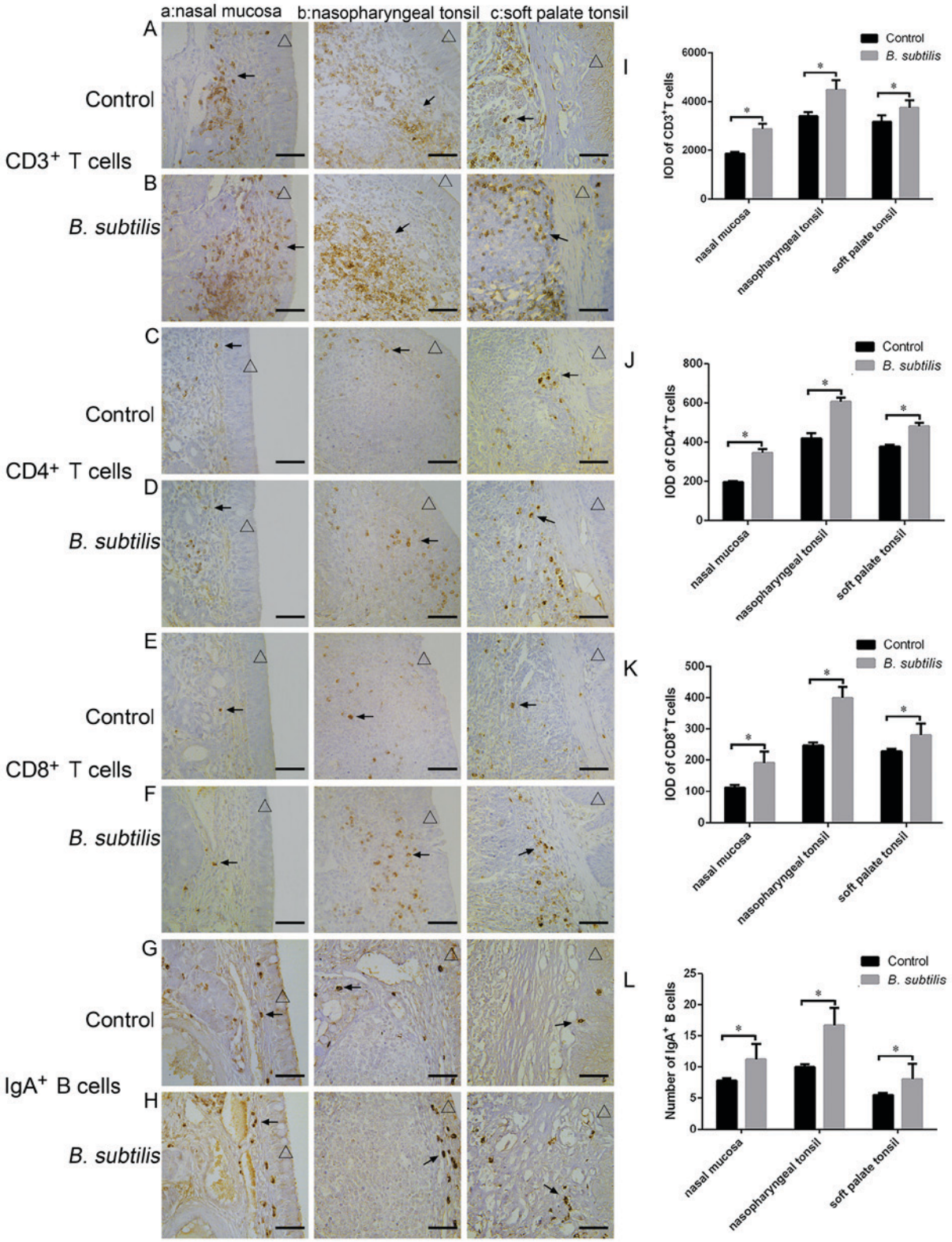

Figure 3. Effects of intranasal administration of *B. subtilis* on $CD3^+$, $CD4^+$, $CD8^+$ T and IgA^+ B cells in the nasal mucosa, nasopharyngeal tonsil and soft palate tonsils of piglets. $CD3^+$ T cells in in (A) control group and (B) *B. subtilis* group. $CD4^+$ T cells in in (C) control group and (D) *B. subtilis* group. $CD8^+$ T cells in in (E) control group and (F) *B. subtilis* group. IgA^+ B cells in in (G) control group and (H) *B. subtilis* group. (a) nasal mucosa, (b) nasopharyngeal tonsils and (c) soft palate tonsils. The triangle represents the epithelium. Scale bar, 100 μm. Bar graphs of the number of (I) $CD3^+$, (J) $CD4^+$ and (K) $CD8^+$ T cells and (L) IgA^+ B cells in in nasal mucosa, nasopharyngeal tonsils and soft palate tonsils. Triangles indicate the epithelium and arrows indicate positive cells. Data are presented as the mean ± standard error of the mean (n=4/group) and the error bars indicate the standard error. $^*P<0.05$. CD, cluster of differentiation; IgA, immunoglobulin A.

tonsil and soft palate tonsil increased significantly following the nasal administration of *B. subtilis* compared with the control ($P<0.05$; Fig. 3I-K).

Effect of B. subtilis on IgA^+ B cells in the nasal mucosa and tonsils. Immunohistochemical staining revealed that the IgA^+ B cells (stained brown) were round or oval in shape and

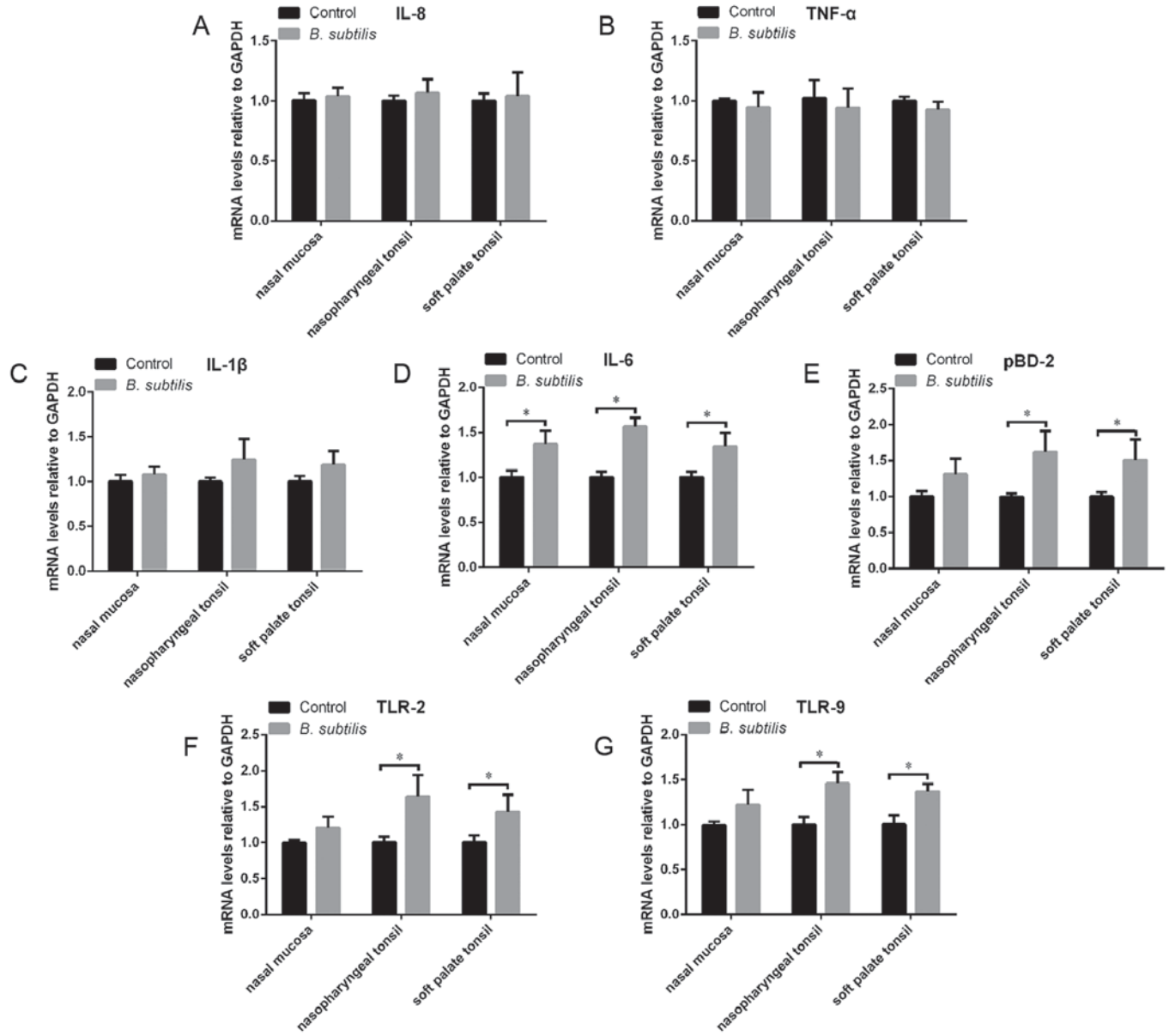

Figure 4. mRNA expression of (A) IL-8, (B) TNF-α, (C) IL-1β, (D) IL-6, (E) pBD-2, (F) TLR-2 and (G) TLR-9 in the nasal mucosa, nasopharyngeal tonsils and soft palate tonsils. Data are presented as the mean ± standard error of the mean (n=4/group) and the error bars indicate the standard error. *P<0.05. IL, interleukin; TLR, toll-like receptor; TNF, tumor necrosis factor; pBD, porcine β-defensin 2.

primarily distributed in the lamina propria of the nasal mucosa, soft palate tonsils and nasopharyngeal tonsils (Fig. 3G-H). Following the intranasal administration of *B. subtilis*, the number of IgA^+ B cells was significantly increased in the nasal mucosa, nasopharyngeal tonsils and soft palate tonsils at 28 days post administration compared with the control (P<0.05; Fig. 3L).

Expression of IL-1β, IL-6, IL-8, TNF-α, pBD-2, TLR-2 and TLR-9 mRNA. RT-qPCR was performed to determine the mRNA levels of IL-1β, IL-6, IL-8, TNF-α, pBD-2, TLR-2 and TLR-9 in the tissue samples (Fig. 4). The expression of IL-6 mRNA in the nasal cavities and tonsils increased significantly following the intranasal administration of *B. subtilis* compared with the control (P<0.05; Fig. 4D). The expression of pBD-2, TLR-2 and TLR-9 mRNA also significantly increased following the administration of *B. subtilis*, but only in the tonsils (P<0.05; Fig. 4E-G). These results indicate that the administration of *B. subtilis* stimulates nasal mucosal immunity. However, no statistical difference in mRNA expression was observed for IL-1β, TNF-α and IL-8 between the *B. subtilis* group and the control group in the nasal mucosa, nasopharyngeal tonsil or soft palate tonsil (Fig. 4A-C).

Protein expression of IL-1β, IL-8, TNF-α, TLR-2, TLR-9. No statistical differences in the protein expression of IL-1β, TNF-α and IL-8 were observed between the *B. subtilis* group and the control group in the nasal mucosa, nasopharyngeal tonsil or soft palate tonsil (Fig. 5A-C). The TLR-2 and TLR-9 protein levels in the nasal mucosa, nasopharyngeal tonsils and soft palate tonsils were significantly increased following intranasal administration of *B. subtilis* (P<0.05; Fig. 5D-I).

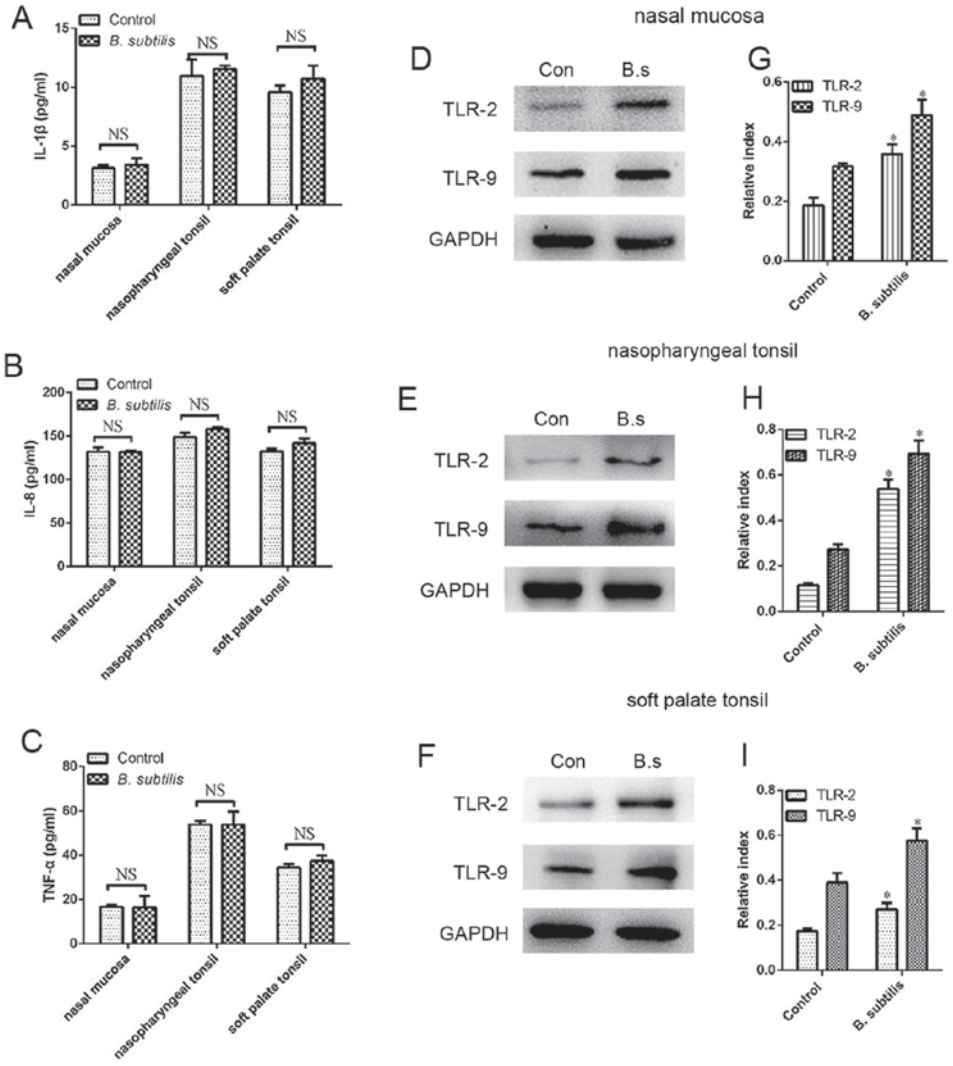

Figure 5. Protein expression of IL-1β, IL-8, TNF-α, TLR-2 and TLR-9 in the nasal mucosa, nasopharyngeal tonsils and soft palate tonsils. ELISA analysis of (A) IL-1β, (B) IL-8 and (C) TNF-α in the nasal mucosa, nasopharyngeal tonsils and soft palate tonsils. Western blot analysis of TLR-2 and TLR-9 protein expression in the (D) nasal mucosa, (E) nasopharyngeal tonsils and (F) soft palate tonsils. TLR-2 and TLR-9 protein expression in the (G) nasal mucosa, (H) nasopharyngeal tonsils and (I) soft palate tonsils were normalized by comparison with GAPDH and the relative index was determined in comparison with the control. Values represent the mean ± standard error of the mean (n=4/group) and the error bars indicate the standard error. *P<0.05. Con, the control group; B.s, the *B. subtilis* group; IL, interleukin; TLR, toll-like receptor; TNF, tumor necrosis factor.

Discussion

The innate immune system represents the first line of defense against pathogen invasion; the innate immunity of the respiratory tract in pigs has previously been studied due to its similarities with humans (30). Innate immunity functions through interactions between immune cells and TLRs, which are primarily expressed on APCs (31). The innate immune system promotes the activation of the adaptive immune system (32) and therefore serves an important role in the body's immune response. TLRs link the innate immune system with specific adaptive immunity (33). TLR-2 has a wide range of ligands, including lipoproteins and lipoteichoic acids from bacteria (34). TLR-9 is able to recognize bacterial DNA, which contains CpG motifs, and activate APCs and native B cells (35). In the present study, treatment with *B. subtilis* significantly increased TLR-2 and TLR-9 protein expression in the nasal mucosa, nasopharyngeal tonsils and soft palate tonsils. The results revealed that immunomodulation induced by *B. subtilis* WB800 primarily depends on extracellular macromolecules TLRs.

Secretory IgA, which is the main antibody for local mucosal immunity, is secreted by B cells (36). It may combine with antigens in the digestive and respiratory tracts and mediate virus neutralization in infected epithelial cells, or adhere to the mucosa to prevent pathogen translocation (37). DCs are the most ubiquitously distributed professional APCs (38) and are widely distributed throughout the secondary lymphoid organs (39). As the primary APCs in the nasal mucosa and tonsils, the protrusions of DCs may extend across the epithelium to uptake antigens and present them to T and B cells (40,41). The maturation of DCs is a complex process during which they express surface molecules and cytokines, which are important for T-cell activation (42). It has previously been suggested that DCs were associated with the proliferation of $CD4^+$ T cells, and that DCs may serve as APCs and upregulate $CD4^+$ T cells in the nasal mucosa (40). The present study demonstrated that *B. subtilis* WB800 treatment significantly increased immune cells, indicating that it enhanced

the local immunity of the piglets. A previous study has demonstrated that pBD-2 inhibits pathogens *in vitro* (27). T cells may produce cytokines that promote the maturation and differentiation of B lymphocytes into antibody producing cells (43,44).

A previous study revealed that probiotics may upregulate the protein expression of pBD-2 in the saliva and intestines of piglets (45). In addition, it has been reported that the administration of probiotics increases the expression of IL-6 in the nasal mucosa, nasopharyngeal tonsil and soft palate tonsil (27,46,47). Based on this previous research, the authors of the present study hypothesized that the protein expression of pBD-2 and IL-6 would increase following the administration of *B. subtilis*. The focus of the present study was local immunity; however, the number of tissue samples was limited. To combat this, the expression of IL-1β, IL-8, TNF-α, TLR-2 and TLR-9 protein was measured in the nasal mucosa, nasopharyngeal tonsils and soft palate tonsils, all of which are key sites for local mucosal immunity. The present study revealed that IL-6 mRNA was significantly increased in the nasal mucosa, nasopharyngeal tonsil and soft palate tonsil following intranasal administration of *B. subtilis*. This may be due to the regulative capability of *B. subtilis* in improving mucosal immune responses by inducing the production of IL-6. No statistical differences were observed in levels of pro-inflammatory factors TNF-α, IL-1β and IL-8 between the *B. subtilis* group and the control. These results indicate that the increase in immune cells and cytokines was not caused by inflammatory reactions. A previous study has reported that *B. subtilis* may effectively modulate the intestinal microbiota by enhancing the proliferation of beneficial bacteria and inhibiting potential pathogens, including *Staphylococcus* and *Escherichia-Shigella,* in the intestinal tract (48). A previous study reported that *B. subtilis* reduced *Salmonella enteritidis* attachment to the surfaces of intestinal epithelial cells (49). Based on this previous research, the authors of the present study hypothesized that *B. subtilis* had the ability to modulate the nasal microbiota and enhance the local mucosal immunity.

In conclusion, intranasal administration of *B. subtilis* increased the number of immune cells in the nasal mucosa, nasopharyngeal tonsils and soft palate tonsils, which enhanced the nasal mucosal and tonsillar immunity of piglets. The present study provides a basis for the further study of intranasal immunization with *B. subtilis* as a mucosal adjuvant. It also lays the foundation for the potential intranasal administration of *B. subtilis* in humans, which may enhance the immune response of human nasal mucosa against respiratory diseases.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 31172302) and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Availability of data and materials

Not applicable.

Authors' contributions

YY and QY conceived and designed the study and wrote the paper. YY, YJ, and JY performed the experiments. YY, YJ, JY, and QY reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

All procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) and followed the National Institutes of Health guidelines for the performance of animal experiments.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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