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Oral administration of *Bacillus subtilis* promotes homing of $CD3^+$ T cells and IgA-secreting cells to the respiratory tract in piglets

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ABSTRACT

Oral probiotics are used to induce immune responses in the intestines to protect against infection. However, oral probiotics may also affect immune responses in other mucosal tissues such as in the respiratory tract. To examine this possibility, we explored the potential of immunocytes to home to the respiratory system after oral administration of *Bacillus subtilis*. The results showed that *B. subtilis* could promote intestinal development and not cause pathological changes in the respiratory tract. Following the oral administration with *B. subtilis*, the number of IgA-secreting cells and $CD3^+$ T cells not only significantly increased in the intestinal tracts but also in respiratory tracts ($P < 0.01$). Moreover, the levels of secretory IgA were significantly higher in the trachea, lungs, ileum, and jejunum after oral *B. subtilis* administration than in the control groups ($P < 0.05$). The mRNA expression of interleukin (IL)-1β, IL-5, IL-6, tumor necrosis factor-α, B cell activating factor, and IgA-inducing protein increased following *B. subtilis* administration ($P < 0.01$) in the trachea, lungs, ileum, and jejunum. These data suggest that *B. subtilis* administration regulates the immune response not only in the intestine but also in the respiratory tract of piglets. Our work highlights a potentially new strategy for promoting respiratory mucosal immunity and may contribute to the design of vaccines with *B. subtilis* as a mucosal adjuvant.

1. Introduction

Mucosal surfaces are frequently exposed to potentially pathological microorganisms. In pigs, the majority of infectious agents, such as influenza A, rhinovirus, and pseudorabies, enter the body through the respiratory mucosal surfaces (Splunter et al., 2018). Further, porcine epidemic diarrhea virus (PEDV) can also be transmitted by the aerosol route(Jia et al., 2015). Boosting the function of the mucosal immune system is considered to be one of the most effective methods to defend against invading organisms as it cuts off the routes of virus transmission and effectively prevents pathogen invasion (Jing et al., 2019).

A number of studies have confirmed that oral probiotics can improve intestinal innate immunity and enhance resistance to intestinal disease (Deng et al., 2013; Huang et al., 2008). However, mucosal immunity in the respiratory tract is also important for protection against infectious disease, but it is difficult to directly enhance immune function in this subdivision of the respiratory system. Nasal immunity can also provide important protection against infectious diseases, but directly boosting immunity at this site is more challenging. So far, the only intranasal vaccine licensed for use in humans is against Influenza A and B(Jia et al., 2015). However, according to the common mucosal immune system (CMIS) theory, oral immunity might induce immune responses not only locally but also in other mucosal sites(Date et al., 2017). In this scenario, activated mucosal B and T lymphocytes could migrate from the inductive site and home to various mucosal effector sites. There is also an increasing body of evidence to suggest that immunostimulation by probiotic bacteria in the gut can enhance immune protection at distal mucosal sites, such as the urogenital and respiratory tracts (Czerkinsky and Holmgren, 2012; Rudin et al., 1998). In this respect, there is much interest in the potential for oral immunity to also elicit protective immune responses in the respiratory tract.

Bacillus subtilis is a common probiotic, which can effectively antagonize pathogenic bacteria (Elshaghabee et al., 2017). The bacterium can form spores which have been safely utilized as natural biological products in humans and animals (Milene et al., 2014). *B. subtilis* spores have also been proven to be an ideal oral vaccine delivery system for presentation of heterologous antigens to the gastrointestinal tract (Ricca et al., 2014). Moreover, *B. subtilis* cannot only drive the development of the gut-associated lymphoid tissue (GALT) but can also improve barrier functions of intestinal epithelial cells (Jia et al., 2015). However,

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whether oral administration of *B. subtilis* could enhance innate immunity in the respiratory tract remains unknown.

In the present study, we explored the immune effects of oral *B. subtilis* administration in the digestive and respiratory tracts. Our study provides perspectives on potential novel strategies and approaches for enhancing respiratory immunity, and the results may form the basis for further studies exploring the use of *B. subtilis* as a mucosal immunopotentiator in oral vaccines.

2. Materials and methods

2.1. Animals and reagents

Twelve (5-day-old) cross-bred Duroc/Landrace/Yorkshire piglets were obtained from the Jiangsu Academy of Agricultural Sciences (Nanjing, China). 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 performing animal experiments. *B. subtilis* WB800 was obtained from the College of Plant Protection, Nanjing Agricultural University (Nanjing, China). *Bacillus subtilis* was grown in LB broth containing 50 μg/ml kanamycin at 37 °C for 12 h, washing the bacterial by 900 *g* centrifugation with PBS. Then suspended *B. subtilis* in PBS to a final concentration of 1×10^8 CFU/ml.

Goat anti-pig IgA, Rabbit anti-pig CD3 monoclonal antibodies were purchased from Abcam. ABC-based system (biotinylated goat anti-rabbit IgG and rabbit anti-goat IgG antibodys) was used as the secondary antibody with DAB as a chromogen was purchased from Boster, Wuhan, China.

2.2. Animal experiments

DLY (Duroc * Landrace * Yorkshire) piglets born on the same day were provided by Jiangsu Academy of Agricultural Sciences (Nanjing, China). 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 performing animal experiments. Twelve male neonatal piglets were randomly divided into two groups (six piglets each group). These piglets delivered healthy and without complications, and their initial weight was similar. The piglets were individually housed in customized compartments, that allowed easy access to the artificial milk a provided by an automated milk dispense. The piglets were housed in Jiangsu Huai'an Pig Farm under a 12 h light/dark cycle with free access to water and food under standard conditions. The piglets were placed in a 37° incubator one week after birth. Then the room temperature was maintained at 32 to 28 °C and relative humidity was controlled at 65% to 75%. The control group was orally administered phosphate-buffered saline (PBS) and the *B. subtilis* group was orally administered *B. subtilis* WB800 spores (10^8 CFU). The piglets were treated with 1 ml aseptic PBS or *B. subtilis* (10^8CFU) suspended in 1 ml PBS by gavage on day 5 of life and were given a booster immunization on day 12. They were euthanized on day 35 of life by intravenous injection of sodium pentobarbital (100 mg/kg) as previously described (Mou et al., 2016). Immediately following euthanasia, the nasal mucosa, nasopharyngeal tonsils, soft palate tonsils, trachea, lung, ileum, and jejunum were collected at necropsy. The samples were fixed in Bouin's fluid for 48 h at 20 °C–25 °C for histological analysis or stored at −80 °C for the detection of interleukin (IL)-1β, IL-5, IL-6, tumor necrosis factor (TNF)-α, B cell activating factor (BAFF), and IgA-inducing protein (IGIP) mRNA using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Tissues were also embedded in paraffin and cut into 5-μm-thick sections for staining.

2.3. Immunohistochemical (IHC) staining of IgA-secreting cells and $CD3^+$ T lymphocytes in the intestine and respiratory tract

Paraffin sections were dewaxed in xylene and rehydrated through decreasing concentrations of ethanol (100%, 95%, 85%, and 75%, each for 1 min) and rinsed in PBS. The endogenous peroxidase activity was quenched by 3% H_2O_2 for 15 min followed by three rinses with PBS at room temperature for 15 min. Subsequently, the sections were treated with 5% bovine serum albumin for 1 h to block non-specific binding. The sections were incubated with rabbit anti-pig CD3 (1:100) or goat anti-pig IgA (1:100) antibodies for 12 h at 4 °C and then incubated with biotinylated goat anti-rabbit IgG or rabbit anti-goat IgG antibodies for 1 h at room temperature. The sections were rinsed three times with PBS and then incubated with horseradish peroxidase (HRP)-labeled streptavidin-biotin complex. After staining with 3,3′-diaminobenzidine, the sections were then sealed with neutral balata. The sections were visualized using a light microscope (Olympus CX23; Olympus Corporation, Tokyo, Japan) at a magnification of ×400. Fifteen fields for each tissue from each piglet were assessed for statistical analysis. Morphometric measurements were obtained using computer-based image analysis software (version X; Media Cybernetics, Silver Springs, MD, USA) to measure the immunopositive optical density for CD3 and IgA. In our experiment, fifteen pictures were randomly selected from each group. The measure of optical density for $CD3^+$T cells and SIgA-secreting cells referred to previous study(Youssef and Salah, 2019).

The SIgA-secreting cells were located mainly in the intestinal lamina propria. The regions that contained SIgA secreting cells were counted under an optical microscope. In our experiment, fifteen pictures were randomly selected from each group and SIgA secreting cells were counted in the same size field of view.

2.4. Enzyme-linked immunosorbent assay (ELISA) for IgA in the mucosa

Tissue samples (50 mg) were placed in 1.5 ml cryogenic vials containing 500 μl PBS and homogenized in Tissuelyser-24 (Jingxin, Shanghai). The supernatant of these homogenates was collected after a 12,000×*g* centrifugation for 10 min at 4 °C for the detection of IgA. The protein concentration of the supernatant was measured using a BCA protein assay kit (Thermo Fisher Scientific). Every sample concentration was same. Sorry we only have a blank control group, and no negative or positive samples. IgA levels in the supernatant were analyzed using an IgA ELISA kit(Jiangsu Meimian industrial Co. Ltd., Jiang/su, China) according to the manufacturer's protocol. In brief, after a 5-fold dilution, pipetted 50 μl of each standard, including zero control, and samples into pre designated wells. Incubated the micro titer plate at 37 °C for 30 min. Kept plate covered and level during incubation. Following incubation, aspirated the contents of the wells. Completely full each well with appropriately diluted 1× Wash Buffer and aspirate. Repeated three times, for a total of four washes. Followed this by sharply striking the wells on absorbent paper to remove residual buffer. Added 50 μl of 1× HRP-Streptavidin solution to each well. Incubated for 30 min at 37 °C. Washed and blotted the wells. Pipetted 100 μl of TMB Substrate into each well. Incubated in the dark at 37 °C for precisely 10 min. Added 50 μl of Stop Solution to each well. Data were acquired on an automated ELISA plate reader at 450 nm immediately using a Tecan plate reader.

2.5. RNA isolation and RT-PCR for IL-1β, IL-6, IL-5, TNF-α, BAFF, and IGIP

Tissue samples (50 mg) were placed in 1.5 ml cryogenic vials containing 1 ml RNAiso Plus and homogenized in Tissuelyser-24 (Jingxin, Shanghai). The tissues were homogenized using 25 mm zirconium oxide grinding balls 3 times at 60 Hz for 30s. Prior to milling, the lung and trachea samples were embrittled using liquid nitrogen, then the lung samples were homogenized 3 times at 60 Hz for 30s. The supernatant of these homogenates was collected after centrifugation at 12,000 ×*g* for

15 min at 4 °C. Total RNA was isolated from homogenized tissues using a TRIzol™ Plus RNA Purification Kit (Thermo Fisher Scientific, Inc.). Total RNA (500 ng) was reverse-transcribed using the PrimeScript™ RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) following the procedures provided by the manufacturers. Approximately 2 μl of diluted cDNA (vol:vol, 1:20) were 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) in accordance with the manufacturer's protocol. The thermocycling conditions for the PCR were 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 31 s. GAPDH was used as a housekeeping gene. The specific primers used for PCR are listed in Table 1. PCR products were analyzed using melting curves, and the results (fold change) were calculated using the $2^{-\triangle\triangle Ct}$ method.

2.6. Statistical analysis

Measurements and cell counts were performed by Image-Pro Plus version 6.0 (Media Cybernetics, Inc.). Student's *t*-tests were used to determine the significance of differences between means. Values are shown as mean ± SD, with P values <0.05 considered to be statistically significant and P values <0.01 considered to show a tendency to be significant in all statistical analyses. All calculations were performed by Statcel3 (OMS, Tokyo, Japan) as an add-in application in Microsoft Excel® (Microsoft, Seattle, WA, USA).

3. Results

3.1. Effects of oral B. subtilis administration on the development of immunity in the intestines and respiratory tract

Peyer's patches (PPs) are aggregated lymphoid nodules that constitute an important part of the immune system; they are important inductive sites for the initiation of adaptive immune responses and function to maximize the immunological barrier of the host (Hiroshi and Satoshi, 2004). One of the most important parameters of PP development is size (Lasa-Saracíbar et al., 2014; Sato et al., 2006). We found that oral administration of *B. subtilis* resulted in enlarged PPs ($P < 0.01$), indicating that *B. subtilis* can promote the development of intestinal immunity in piglets (Fig. 1).

In addition, the morphological structure of the epithelial tissue remained intact after oral administration of *B. subtilis* (Fig. 2A–D), indicating that *B. subtilis* administered via this route did not cause pathological changes in tissues. Moreover, no inflammatory injury was observed in the intestinal and respiratory tissues of piglets from either of the two groups.

Table 1
Primers used for qRT-PCR.

Gene		Sequence (5′-3′)
BAFF	Forward	AACCGTGGTCAAAGAAAC
	Reverse	CAGGAATTATTGGGTAGTGT
IGIP	Forward	TTGCTGTCATGTTCTCC
	Reverse	CATTTGCTTGGTTTCCAC
IL-1β	Forward	TCATCGTGGCAGTGGAGAAGC
	Reverse	TCT GGG TAT GGC TTT CCT TAG
GAPDH	Forward	TCATCATCTCTGCCCCTTCT
	Reverse	GTCATGAGTCCCTCCACGAT
IL-5	Forward	CGTTAGTGCCATTGCTGT
	Reverse	GTTCCCATCGCCTATCAG
IL-6	Forward	ACCCTGAGGCAAAAGGGAAAG
	Reverse	CAGGTGCCCCAGCTACATTA

3.2. Effects of B. subtilis on $CD3^+$ T cells in the intestines and respiratory tract

We observed that $CD3^+$ T cells were widely distributed in the lamina propria of the nasal mucosa, soft palate tonsils, and nasopharyngeal tonsils (Fig. 3A–F). Cells staining positive using anti-CD3 antibody appeared brown. Oral *B. subtilis* administration resulted in a significantly augmented number of $CD3^+$ T cells in the intestinal and respiratory tracts compared with control group ($P < 0.05$) (Fig. 3G–H).

3.3. Effects of B. subtilis on IgA-secreting cells in the intestines and respiratory tract

IgA-secreting cells were mainly detected in the submucosa, and the number of IgA-secreting cells was significantly ($P < 0.01$) increased in the intestinal and respiratory tracts, including the nasal mucosa, nasopharyngeal tonsils, soft palate tonsils, trachea, and lungs, after oral administration of *B. subtilis* (Fig. 4A–I).

Expression of the IgA protein in different tissues was detected by ELISA. Oral *B. subtilis* administration resulted in significantly ($P < 0.01$) increased IgA protein levels in the trachea, lungs, ileum, and jejunum compared with control group (Fig. 4H).

3.4. Expression of IL-1β, IL-6, IL-5, TNF-α, BAFF, and IGIP mRNA

To investigate the effect of oral *B. subtilis* administration on IL-1β, IL-6, IL-5, TNF-α, BAFF, and IGIP expression, RT-PCR was performed on total RNA extracted from different tissues. mRNAs for these genes were detected in the trachea, lungs, ileum, and jejunum. The expression levels of IL-1β, IL-6, IL-5, TNF-α, BAFF, and IGIP mRNA in the intestinal and respiratory tracts increased ($P < 0.01$) following the oral administration of *B. subtilis* compared with the oral administration of PBS (Fig. 5A–F). The expression of IL-6 mRNA also remarkably ($P < 0.01$) increased following the administration of *B. subtilis* in the lungs and ileum, but not in the trachea and jejunum. These results indicate that the oral administration of *B. subtilis* can stimulate mucosal immunity in the intestinal and respiratory tracts.

4. Discussion

Enhancing respiratory tract immunity of piglets can greatly improve the survival rate of piglets. There are a large number of reports on how to immunize piglets through intestinal tract or injection, but there are few studies on how to improve nasal immunity against respiratory pathogens. The administration of nasal immunization involves additional costs in terms of the production of the spray device and the appropriate training of staff. Thus, we need to explore other immunological ways to protect the respiratory tract. Orally immunization could induce antigen-specific B and T cells to home to all mucosal surfaces through the common mucosal immune system(Iijima et al., 2010). In addition, compared with systemic immunity, oral immunity can be induced protectively on the mucosal surface where infection begins, and is convenient for administration(Foss and Murtaugh, 2000). Here, we investigated the ability of orally administered *B. subtilis* to provide immunity at mucosal surfaces that might assist in protecting pigs against important pathogens.

The main effector of respiratory mucosal immunity was SIgA , it exerts its functions in defense through the agglutination and neutralization of bacteria, viruses, and toxins; furthermore, it stimulates T cells and B cells, enhancing mucosal immunity (Austin et al., 2003). Studies have shown that IgA-secreting cells lacking gut homing ability in the intestine provide poor protection against intestinal pathogenic virus infection (Kuklin et al., 2001). It was shown previously that oral administration with cholera vaccine promotes homing of IgA^+ memory B cells to the respiratory tract; however, this study investigated that these homing memory B cells were in peripheral blood but not in local

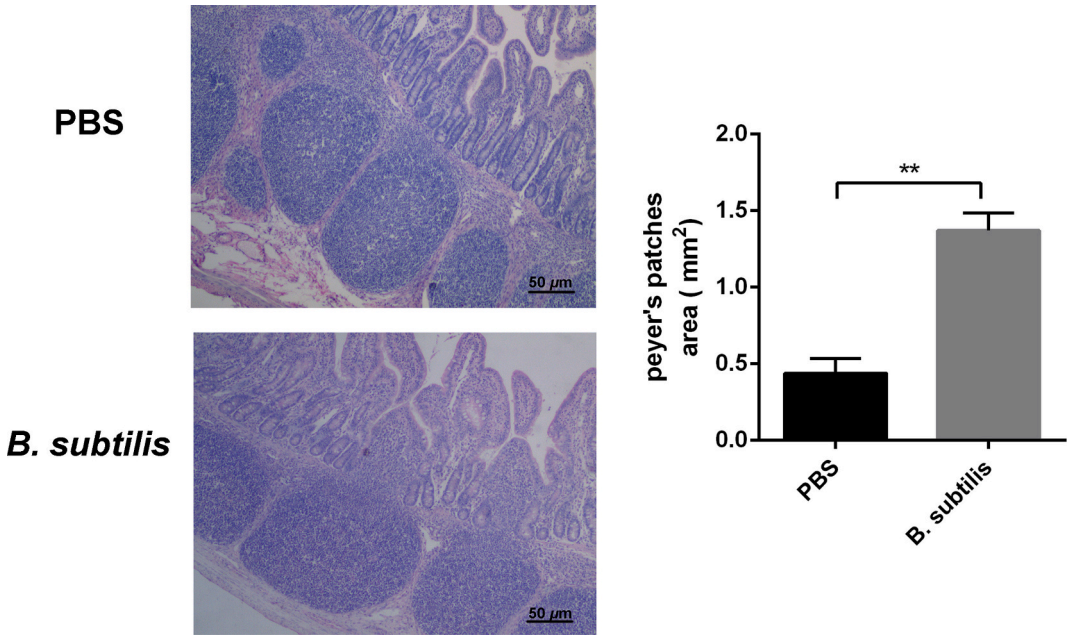

Fig. 1. Oral administration of *B. subtilis* promotes the development of intestinal immunity in piglets.
Piglets were given PBS or 1×10^8 CFU *B. subtilis* on day 5 of life and then the procedure was repeated seven days later. Small intestines were removed and hematoxylin and eosin (H&E) staining was performed. The size of PPs was analyzed and statistical analysis was performed. Data are presented as the mean ± SD error of the mean ($n = 6$/group) and the error bars indicate the standard error. $^*P < 0.05$; $^{**}P < 0.01$.

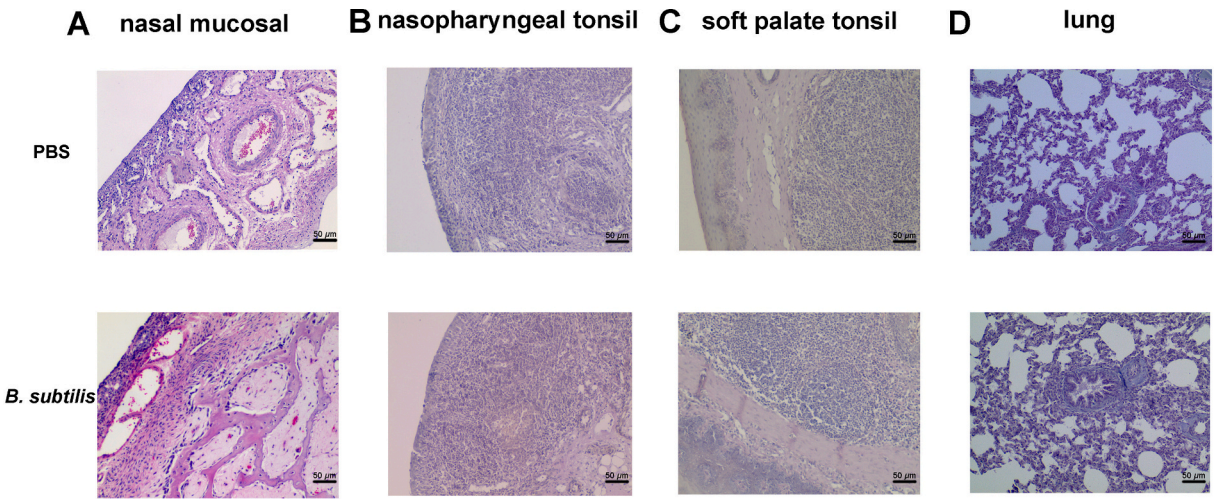

Fig. 2. Effects of oral administration of *B. subtilis* in the respiratory tract of piglets.
H&E staining was performed on control samples and *B. subtilis* samples taken from the (A) nasal mucosa, (B) nasopharyngeal tonsil, (C) soft palate tonsil, and (D) lungs.

tissue (Splunter et al., 2018). In this study, we demonstrated that oral administration of *B. subtilis* successfully enhanced the number of IgA-secreting cells and IgA levels in the respiratory tract. This suggests to a certain extent that oral administration of *B. subtilis* can promote the development of the respiratory mucosal immune system and play an effective role in protecting the respiratory system from invading pathogens.

The mucosal immune system mounts immune responses through cells residing in mucosal compartments (Naafs, 2018). T lymphocytes residing in the mucosa play important roles in mucosal immunity (Shi et al., 2017). Mucosal T lymphocytes responses have been described after oral, nasal, rectal or vaginal immunization(Hyland et al., 2004). In several animal models of respiratory viral infections, T lymphocytes have been shown to be critical for immune clearance of pathogens (Klavinskis et al., 1996; Staats et al., 2001). Some adjuvants such as cholera toxin and related enterotoxin combined with soluble proteins and peptides can promote the development of mucosal T lymphocytes (Bender et al., 1992). Previous study has shown that oral administration of *B. subtilis* can result in increased levels of $CD3^+$ T cells in the small intestine and improve cellular immunity (Jing et al., 2019). In this study, we confirmed a similar immune response in respiratory tract. The results suggest that *B. subtilis* could elicit both humoral and cellular immune responses in the intestinal and respiratory tracts when administered via the oral route.

Cytokines are intercellular molecular messengers that exert their principal effects on other cells in the local milieu (Duque and Descoteaux, 2014; Maini, 1996). Cytokines regulate immune responses by binding to corresponding receptors to regulate cell growth,

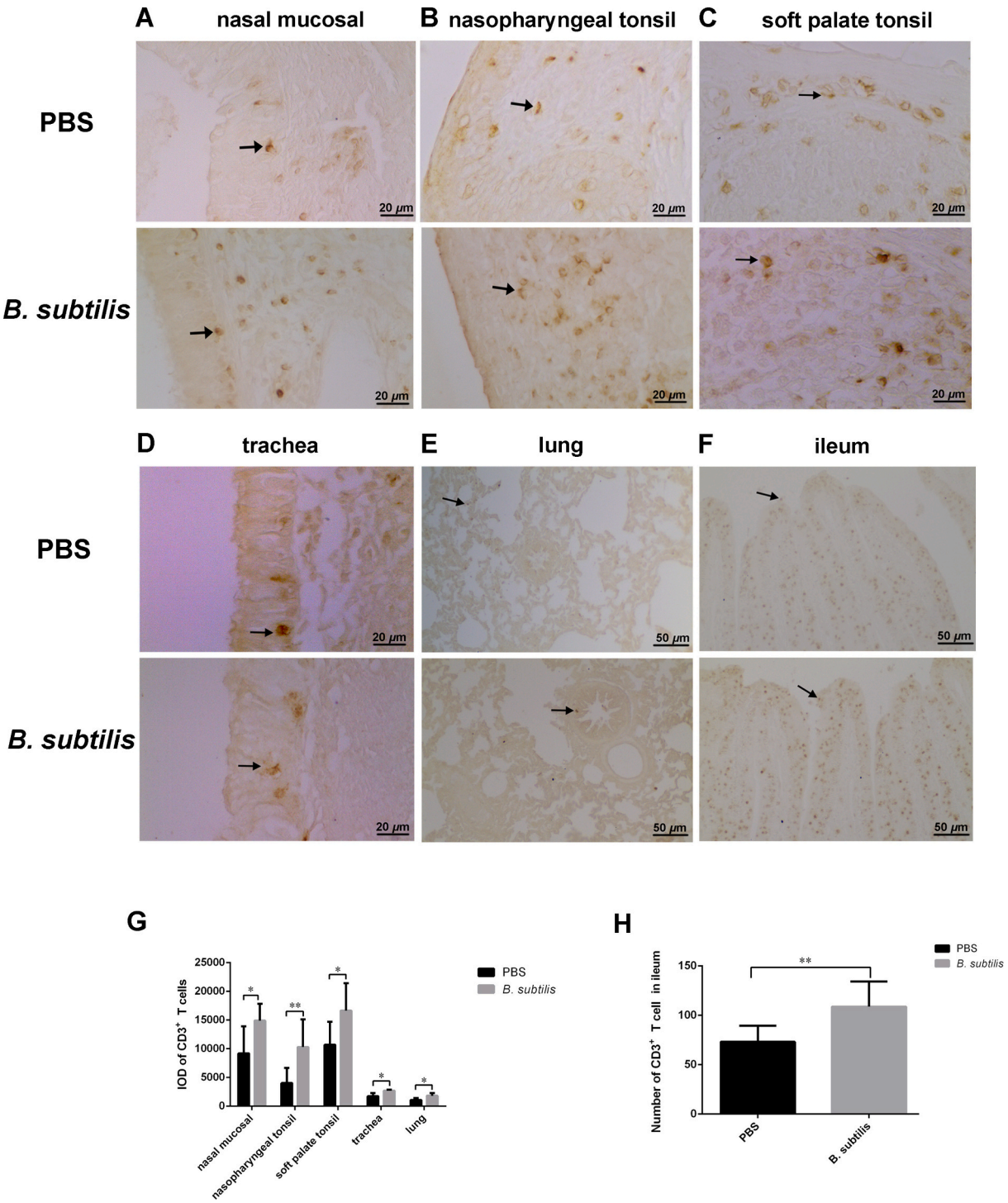

Fig. 3. Effects of oral administration of *B. subtilis* on $CD3^+$ T cells in the nasal mucosa, nasopharyngeal tonsil, soft palate tonsils, trachea, lung, and ileum of piglets. Immunohistochemistry was used to detect $CD3^+$ T lymphocytes in the control and *B. subtilis* groups; (A) nasal mucosa, (B) nasopharyngeal tonsils, (C) soft palate tonsils, (D) trachea, (E) lungs, (F) ileum. Data are presented as the mean ± SD error of the mean (n = 6/group) and the error bars indicate the standard error. $^*P < 0.05$; $^{**}P < 0.01$.

differentiation, and other physiological processes. Therefore, they are effective indicators for assessing the extent of immune responses after stimulation. In this study, we found that mRNA levels of IGIP and BAFF were significantly increased after oral administration of *B. subtilis*. Previous studies revealed that IGIP and BAFF promote T cell-independent mucosal IgA responses (Austin et al., 2003; Ren et al., 2016). The closely related TNF family ligands BAFF and APRIL function in the generation and maintenance of mature B lymphocytes (Sonia et al., 2015). These cytokines further enhance IgA responses by emitting survival signals and/or inducing plasma cell differentiation and IgA secretion (Boyaka, 2017). In the present study, IL-1β and TNF-α mRNAs were significantly increased in the trachea, lungs, ileum, and jejunum following oral administration of *B. subtilis*. IL-1β is one of the first pro-inflammatory cytokines to be secreted during infection, and its levels can be increased by TNF-α; it is also plays a central role in the regulation of immune responses (Sanchez-Mu Oz et al., 2008). Th2 cytokines, such

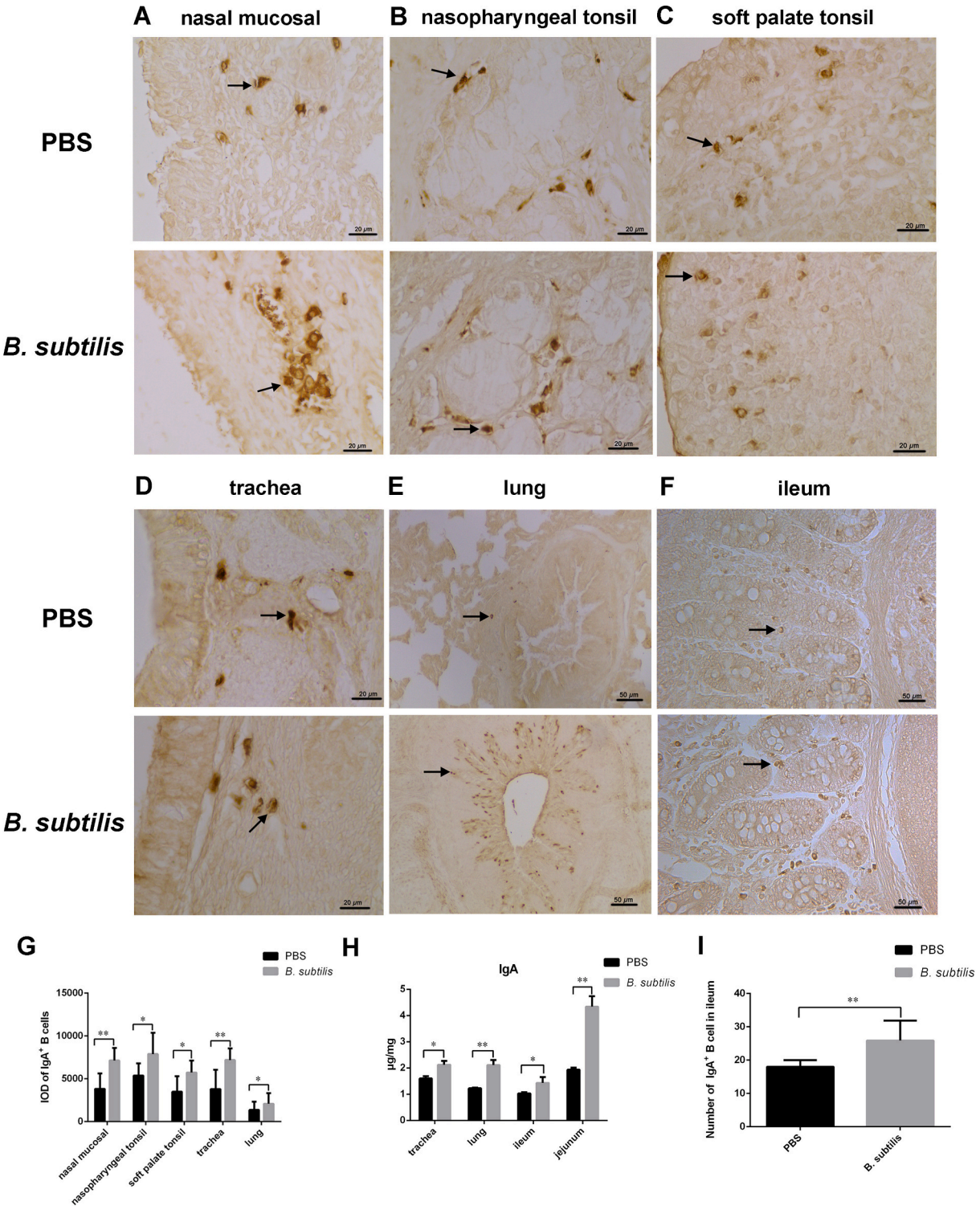

Fig. 4. Effects of oral administration of *B. subtilis* on IgA-secreting cells in the nasal mucosa, nasopharyngeal tonsil, soft palate tonsils, trachea, lung, and ileum of piglets.

IgA-secreting cells were detected by immunohistochemistry; (A) nasal mucosa, (B) nasopharyngeal tonsils, (C) soft palate tonsils, (D) trachea, (E) lung, (F) ileum. Trachea, lung, ileum, and jejunum were homogenized in PBS. Supernatants were collected and tested for (H) IgA by ELISA. Data are presented as the mean ± SD error of the mean (n = 6/group) and the error bars indicate the standard error. *$P < 0.05$; **$P < 0.01$.

as IL-5 and IL-6 meanwhile, are necessary for the differentiation of immature B cells into IgA-secreting cells (Ren et al., 2016). Our data showed that oral administration with *B. subtilis* regulated and enhanced immunity by upregulating these cytokines.

Increases in T cells, IgA-secreting cells, and in the secretion of cytokines are crucial for mucosal immune responses (Montilla et al., 2004). The observation of these phenomena in the respiratory tract in this study shows that *B. subtilis* can augment the immune response of the respiratory mucosa. The oral method of immunization tested in this experiment will result in a portion of the bacteria entering the nasal

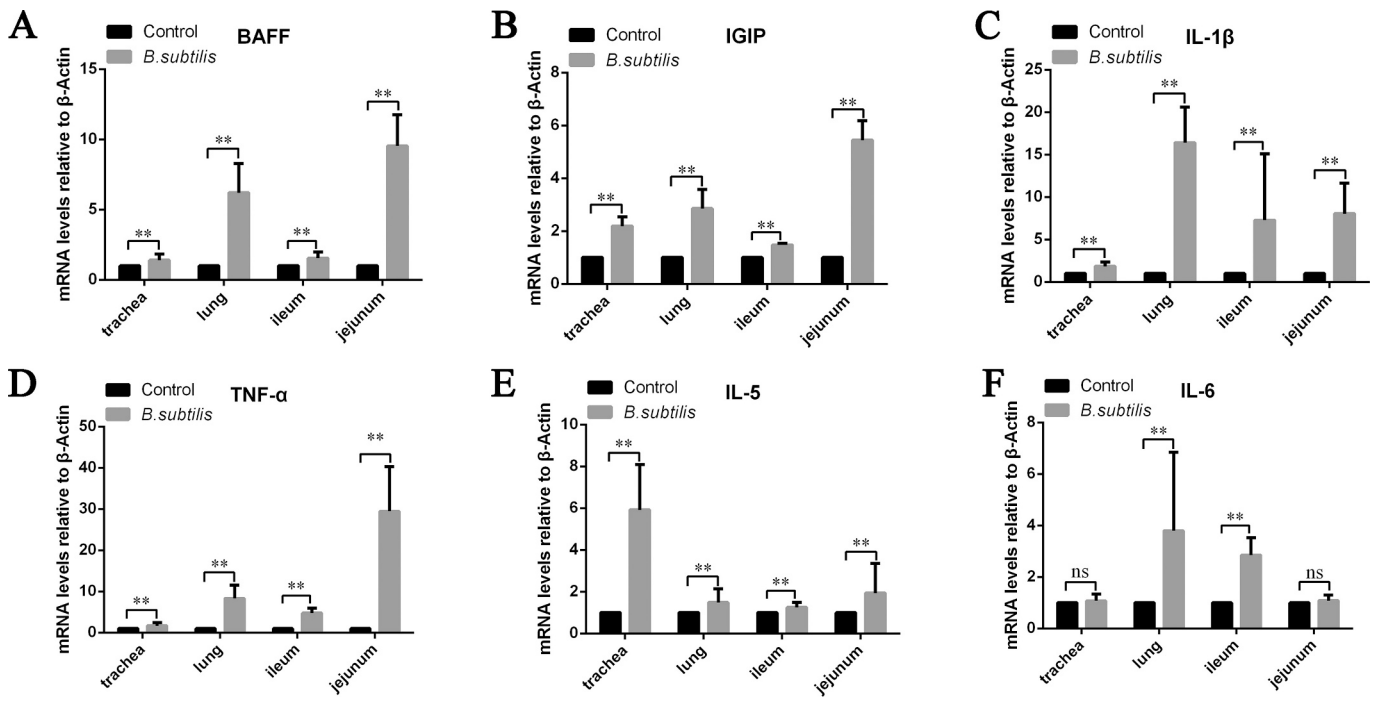

Fig. 5. The determination of cytokine responses by RT-qPCR.
mRNA was extracted from trachea, lung, ileum, and jejunum. The expression of (A) BAFF, (B) IGIP, (C) IL-1β, (D) TNF-α, (E) IL-5, and (F) IL-6 was detected by RT-qPCR. Data are presented as the mean ± SD error of the mean (n = 6/group) and the error bars indicate the standard error. *$P < 0.05$; **$P < 0.01$.

cavity. Thus, immune reactions in the respiratory tract may be partially elicited by spores at this site, but since the numbers of spores reaching the nasal cavity remain low, this is unlikely to be enough to trigger a strong immune response.

In conclusion, oral administration of *B. subtilis* increased the number of immune cells in the nasal mucosa, nasopharyngeal tonsils, soft palate tonsils, trachea, lung, and ileum, which together resulted in enhanced piglet immunity. The present study provides a basis for further studies using oral immunization to boost respiratory immunity in piglets and for developing new approaches to promote immune function in these animals.

Ethics approval and consent to participate

All animal experiments 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 performing animal experiments.

Availability of data and materials

The data can be provided by the corresponding author on reasonable request.

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Author contributions

PZ study conception and design, data analysis and interpretation, manuscript writing. LH performed most of the immunization and EZ helped in the animal experiment. QY study conception and design, financial support, administrative support, data analysis and interpretation, manuscript writing, final approval of the manuscript.

Consent for publication

Informed consent was obtained from all individual participants included in the study.

Declaration of Competing Interest

The authors declare no competing financial interests.

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