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Effects of porcine IL-17B and IL-17E against intestinal pathogenic microorganism

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ABSTRACT

The interleukin-17 (IL-17) family plays a critical role in host defense, allergic reactions, and even tumorigenesis on different mucous membranes. IL-17 family has been cloned in human and mouse, as well IL-17A, IL-17 F in swine. So far, current knowledge on the cloning and biological functions of porcine IL-17B (poIL-17B) and porcine IL-17E (poIL-17E) is limited. In this study, poIL-17B and poIL-17E, mainly expressed in intestine, were cloned and characterized. Expression of poIL-17B and poIL-17E was upregulated after pathogenic microorganism infection. Moreover, the significant enhanced expression of antibacterial peptides PR-39 and pBD-1 was observed when poIL-17B and poIL-17E were over-expressed in the small intestinal epithelial cell line IPEC-J2. This demonstrated that poIL-17B and poIL-17E might have anti-infective capability. Pathogens infection data showed that pathogens could up-regulate poIL-17B/E expression levels. After stimulating the cells with the pathogen, continued with probiotics, the expression of poIL-17B/E was down-regulated. Meanwhile, the induced expression of poIL-17E was greater than that of poIL-17B. Invasion data indicated that poIL-17B and poIL-17E both could inhibit effectively pathogenic microorganism, while inhibitory capability of poIL-17B was stronger than that of poIL-17E. Therefore, poIL-17B and poIL-17E both could be important members against intestinal infection in the porcine IL-17 family. This study provided a theoretical basis for the prevention of intestinal diseases in pigs and thus achieved healthy farming.

1. Introduction

IL-17 has a cysteine knot that is formed by the interaction between four conserved cysteine residues (Hymowitz et al., 2001). It has been functionally characterized in vertebrates and invertebrates (Huang et al., 2015). Most mammalian IL-17 family members includes: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17 F, and its receptor family includes IL17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE (Gaffen, 2009). Members of the IL-17 family play an important role in host defense, allergic reactions, and tumor production on different mucosa (Saenz et al., 2010). IL-17A and IL-17 F could induce strong inflammatory responses. IL-17B, IL-17C and IL-17E are expressed by epithelial cells (Reynolds et al., 2015; Song et al., 2011). Therefore, they are very important for intestinal immune response where it regulates the expression of many innate immune genes. Research on IL-17 family could provide reference for the treatment of gastrointestinal pathogens in animal husbandry.

IL-17E (also known as IL-25) is a member of the IL-17 cytokine family. They are high levels of IL-17E mRNA in polarized Th2 cells (Fort et al., 2001). IL-17E is produced from a variety of cellular sources, such as epithelial cells, endothelial cells, eosinophils and basophils. They can promote the chemotactic activity of Th2 cytokines to participate in inflammatory responses (Saenz et al., 2010). IL-17E is also dependent on type 2 cytokines to induce rapid worm excretion (Fallon et al., 2006). IL-17E plays an important role in the intestinal anti-parasitic. Th2 cytokine expression increases and promotes worm clearance from the intestine after IL-17E injection (Zhao et al., 2010). IL-17E is a key regulator of intestinal mucosal inflammation. It can promote type 2

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immune response, while inhibiting Th1 and Th17 responses. Hong HY et al. found that the expression of IL-17E was significantly increased in nasal tissue of patients with chronic sinusitis and nasal polyps, indicating that IL-17E promoted sinusitis caused by Th2 cells (Hong et al., 2018). The expression of IL-17E was down-regulated in inflammatory bowel disease and colon tissue in inflammatory mice induced by dextran sulfate sodium (DSS) (Su et al., 2013). In vivo injection of recombinant IL-17E could suppress acute colitis induced by DSS, trinitrobenzene sulfonic acid (TNBS) or peptidoglycan (PGN) (McHenga et al., 2010). In summary, IL-17E has a dual immunomodulatory function that can both promote inflammation and inhibit the spread of inflammation.

There is less research on IL-17B, which has pro-inflammatory effects under certain conditions. IL-17B is highly expressed in chondrocytes, connective tissue cells, neuronal cells, colorectal cancer cells and breast cancer cells (Al-Samadi et al., 2016; Ferretti et al., 2016; Lee et al., 2015). Studies have reported that IL-17B plays protective immunity during infection (Reynolds et al., 2015). IL-17RE, which is composed of IL-17RB and IL-17RA heterodimer (Rickel et al., 2008). However, IL-17RB is a receptor of IL-17B (Yao et al., 1995). This suggested that there might be unexplored and unique functions of IL-17B, although the affinity of IL-17B for IL-17RB is weaker compared to IL-17E (Lee et al., 2001).

The intestinal epithelial cell is the first barrier to interact with the intestinal bacteria (Peterson and Artis, 2014). Thus, the study on the intestinal infection was mostly performed on the intestinal epithelial cells. The intestinal porcine epithelial cell line IPEC-J2 (intestinal porcine epithelial cells from jejunum) was isolated from neonatal piglet mid-jejunum in 1989 (Berschneider, 1989). Porcine intestinal epithelial cells are more closely mimic human physiology than analogous rodent cell lines (e.g. IEC-6 or IEC-18), which is important in studies of zoonotic infections; in addition, they provide specificity to study porcine-derived infections (Brosnahan and Brown, 2012). IPEC-J2 cells have been used for immunomodulatory studies of a variety of gastrointestinal commonly used pathogens or probiotics (Botić et al., 2007), including *Escherichia coli, Enterococcus faecium* and *acidophilus Lactobacillus acidophilus, Bacillus subtilis, Salmonella choleraesuis, Saccharomyces cerevisiae*, etc. In this paper, all the infection analyses were performed in epithelial cell line IPEC-J2. Here the microbial stains (probiotics or pathogenic microorganisms), including *Pseudomonas aeruginosa, Streptococcus pyogenes, Lactobacillus reuteri, Saccharomyces cerevisiae* and so on, were chosen to study the connections between porcine IL-17B (poIL-17B)/ porcine IL-17E (poIL-17E) and intestinal infection.

In livestock, neither poIL-17B nor poIL-17E in livestock has been cloned and characterized. In this paper, it was first time to clone poIL-17B and poIL-17E. To investigate the function of poIL-17B and poIL-17E in swine, we over-expressed poIL-17B and poIL-17E in epithelial cell line IPEC-J2. We found that the expression of antimicrobial peptides (especial PR-39 and pBD-1) was enhanced significantly in poIL-17B and poIL-17E over-expressed cells. In addition, we investigated the connection among microbes (pathogenicity and probiotics), poIL-17B/E and pathogenic microbial invasion rates. Our data indicated that both poIL-17B and poIL-17E played a protective role in the porcine intestinal immune system when a series of microorganisms were used to stimulate IPEC-J2 cells.

2. Materials and methods

2.1. Collection of cells and porcine tissues

The intestinal porcine epithelial cell line IPEC-J2 was a gift from Yongliang Zhang of the College of Animal Science, South china of agricultural university. Porcine tissue samples including small intestine, liver, lung, muscle, trachea, brain, skin, kidney, stomach, testis, heart and spleen, were collected from three four-day-old piglets (landrace). Piglets were housed in controlled environment and used in accordance with the protocol (SCAU-AEC-2010-0416) which was approved by the Institutional of Animal Ethics Committee (IAEC) of South China Agricultural University. The isolated tissues were immediately frozen in liquid nitrogen pending RNA extraction until use.

2.2. Cloning of poIL-17B and poIL-17E

The expressed sequence tag (EST) sequences (EW422662.2, CF366793.1, BQ604493.1 and BX668894.2) were identified by blasting the human IL-17B mRNA (NM_014443.2) in Genbank *Sus scrofa* EST database (Sus (taxid: 9823)). The DNA sequences were assembled by DNAman 8.0 to predict the cDNA sequence of *poIL-17B*, and the specific primers based on the predicted open reading frame were designed. Then the cDNA of *poIL-17B* was amplified from the porcine small intestine tissue cDNA library using IL-17B-F and IL-17B-R primers. Finally, the PCR products were cloned into pMD-18 T vector (Takara, Dalian, China) for sequencing.

Search the nucleotide database of NCBI for the mRNA sequences of human IL-17E (NM_172314.1), murine IL-17E (NM_080729.3), and the predicted mRNA sequences of porcine IL-17E (XM_005666258.3), hircine IL-17E (XM_018054603.1), and bovine IL-17E (XM_015473071.1). Then run alignment and designed degenerate primers by Primer Premier 5.0. The cDNA of porcine IL-17E was amplified from the porcine small intestine tissue cDNA library using IL-17E-F and IL-17E-R primers. Finally, the PCR products were cloned into pMD-18 T vector for sequencing.

2.3. Tissue distribution of the poIL-17B/E gene

To validate the tissue distribution of the *poIL-17B/E* gene in swine, the specific primers for real-time PCR were designed by Primer Premier 5.0 and NCBI primer BLAST program. They were given in Table 1, β-actin (as control the gene). Trizol (Thermo Fisher Scientific, USA) was used to isolate total RNA from cells. Purity RNA samples used as templates for reverse transcription (Beyotime, Shanghai, China). The reaction process was conducted in a Bio-rad CFX96 system (Bio-Rad, Hercules, CA, USA). The Ct values of poIL-17B/E were normalized to those of β-actin to gain the ΔCt and the expression of IL-17B/E in various tissues were represented as $2^{-\Delta\Delta Ct}$. Results were representative of three biological replicates.

2.4. Expression of IL-17B and IL-17E in intestinal immune cell populations

Based on high levels of the *poIL-17B/E* gene in intestine, expression of IL-17B/E in intestinal immune cell populations were further analyzed. Mesenteric lymph nodes were collected aseptically and placed in RPMI-1640 medium. They were first minced with scissors before pressing them through stainless steel screens (100 mesh) of a cell collector. All cell suspensions were filtered through steel screens and washed twice before using. Cell viability after treatment exceeded 90% as assessed by Trypan blue dye exclusion. Cells were washed twice and then incubated with fluorescence-conjugated antibodies respectively prepared for flow cytometric separated. The fluorescence-labeled cells (CD45 + CD3 + T cells, CD45 + CD19 + B cells) were isolated with FACSAria (BD Biosciences, San Jose, CA) after excluding residual erythrocytes, debris, doublets and dead cells by forward scatter, side scatter and propidium iodide gating, for detection of IL17B/E expression (the method of IL17B/ E detection was same as section 2.3).

2.5. Construction of poIL-17B/E over-expressed in IPEC J2 cells line

The specific primers were designed by Primer Premier 5.0 after analyzing the possible restriction enzyme cutting site and Kozak sequence was added at upstream of the initiation codon to enhance eukaryotic expression. Then the PCR reaction was carried out by using

Table 1
Primers for PCR reactions.

Primer name	Sequence	Primer used for
IL-17B-F1	ACGAGGCGGCATCTGGGCT	Amplify poIL-17B
IL-17B-R1	CCTTCGTCTCTGCCGGAAT	cDNA
IL-17B-F2	CGGAATTCCAGCCCAGGAACCCCAAA	Construction of
IL-17B-R2	CCCAAGCTTGAAGATGCAGGTGCAGCC	poIL-17B prokaryotic expression vector
IL-17B-F3	CCGCTCGAGACCACCATGGACTGCCTGCA	Construction of
IL-17B-R3	CCCAAGCTTGAAGATGCAGGTGC	poIL-17B Eukaryotic Expression Vector
IL-17B-qF	CCAAGAGGAAGTGTGAGG	poIL-17B qPCR
IL-17B-qR	TCGTGGTTGATGCTGTAG	
IL-17E-F1	ATAAAACCAGGACTCCTAAACTGCT	Amplify poIL-17E
IL-17E-R1	GCACCCATGTCAAAAGTAACTACACC	cDNA
IL-17E-F2	CATGCCATGGGCTTTCCATTTGTGGTCCA	Construction of
IL-17E-R2	CCGCTCGAGGGCCATCACACGGGGCCGCAC	poIL-17E prokaryotic expression vector
IL-17E-F3	CCGCTCGAGACCACCATGTACCAGGC	Construction of
IL-17E-R3	CCCAAGCTTGGCCATCACACGGG	poIL-17E Eukaryotic Expression Vector
IL-17E-qF	AAGGCGAGTGAAGACGGACC	poIL-17B qPCR
IL-17E-qR	AACGAGCGTGGTACAAGT	
M13F	CGCCAGGGTTTTCCCAGTCACGAC	Universal primer
M13R	AGCGGATAACAATTTCACACAGGA	for pMD18
T7F	TAATACGACTCACTATAGGG	Universal primer
T7R	TGCTAGTTATTGCTCAGCGG	for pET28a
T7F	TAATACGACTCACTATAGGG	
BGHR	TAGAAGGCACAGTCGAGG	Universal primer
β-actin-F	CAGGTCATCACCATCGGCAACG	for pcDNA3.1
β-actin-R	GACAGCACCGTGTTGGCGTAGAGGT	reference primers
IL-1β-qF	CAAAGGCCGCCAAGATATAA	for qPCR IL-1β qPCR
IL-1β-qR	GCAGCAACCATGTACCAACT	
IL-8-qF	TGCAGAACTTCGATGCCAGT	IL-8 qPCR
IL-8-qR	ACAGTGGGGTCCACTCTCAA	
IL-10-qF	TAGGGTGTGCCCTATGGTGT	IL-10 qPCR
IL-10-qR	GGGTGGGTAGGCTTGGAATG	
PR-39-qF	TTCAAGGAGAACGGGCGAG	PR-39 qPCR
PR-39-qR	TGGCAAATATGGGGGTCGGG	
pBD-1-qF	TCCTTGTATTCCTCCTCA	pBD-1 qPCR
pBD-1-qR	ACACGCCTTTATTCCTTA	
pBD-2-qF	TCCGACCACTACATATGTGCC	pBD-2 qPCR
pBD-2-qR	CTTGCCACTGTAACAGGTCC	
pBD-3-qF	GAAGTCTACAGAAGCCAAAT	pBD-3 qPCR
pBD-3-qR	GGTAAACAAATAGCACCATAA	

pMD18-poIL-17B/E as template and cloned into vector pcDNA3.1 (Takara, Dalian, China). The plasmids without endotoxin were extracted using SanPrep endotoxin free plasmid extraction kit (Sangon Biotech, Shanghai, China) for lipofection transfection. Liposomes were used to transfect 3 clone cells respectively, according to the procedure of Lipofectamine 2000 Kit (nvitrogen, USA). Cellular RNA was extracted 48 h after transfection. The expression of poIL-17B, poIL-17E, IL-1β, IL-8 and IL-10, as well as PR-39, pBD-1, pBD-2 and pBD-3 were detected by RT-qRCR in each clone (3 repeats for each clone). The primers were respectively given in Table 1.

2.6. Co-culture of IPEC J2 with microbe

Various pathogens and probiotics which were collected by our laboratory were used to stimulate IPEC J2 cells in this paper, including *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* PAO1, *Streptococcus pyogenes* ATCC 19615, *Enterohemorrhagic Escherichia coli* (EPEC) CICC 10411, *Enterohemorrhagic Escherichia coli* (EHEC)ATCC 35150, *Salmonella enterica serovar Typhimurium* LT2, *Candida parapsilosis* ATCC 20019, *Lactobacillus reuteri* DSM 20016, *Streptococcus thermophilus* ATCC 14485, *Saccharomyces cerevisiae* CICC 1562, *Shigella sonnei* CMCC 51592 and *Candida albicans* ATCC 10231.

Microorganisms were inoculated into 5 mL suitable fluid nutrient medium, cultured at 37 °C with 180 rpm. After overnight culturing, microbe cells were collected, and washed for three times by using PBS, and adjusted to 1×10^8 CFU/mL in DMEM/F12. IPEC J2 cells were maintained in Dulbecco's MEM nutrient mix F12 (DMEM-F12) (1:1) (Gibico, Guangzhou, China) with 10% (v/v) FCS (Gibico, Guangzhou, China) at 37 °C in 5% CO_2. Before adding microbe, IPEC-J2 cells were resuspended by trypsinization with 0.25% trypsin-EDTA, and seeded into 24-plates at 1×10^5 cells/well without antibiotics. When the cell adherence rate reaches 75–85 % after 8–12 h, microbes were added for stimulation. In the infection model, the cell suspension was added to wells at 1 or 10 of MOI (multiplicity of infection, Number of microbe/number of IPEC J2 cells). Then it was co-cultured for 6 or 12 h, before the gene expression analysis. After the treatment, the IPEC J2 cells were washed for three times with sterile PBS and total RNA of the cells was isolated as described above. The expression analysis of poIL-17B/E or β-actin was performed by the RT-qPCR described above. The value also calculated according to the formula $2^{-\Delta\Delta Ct}$, which ΔCt is the difference in cycle threshold (Ct) value between IL-17B/E and β-actin. Results were representative of three biological replicates.

2.7. Detection of invasion rate

pcDNA3.1-poIL-17B, pcDNA3.1-poIL-17E or pcDNA3.1 transfected IPECJ2 cells were co-cultured with microbe (MOI = 10) for 12 h. Cells were shaken vigorously on the oscillator for 10 min, and microscopic examination confirmed that there were no adherent microorganisms on the cell surface. Then, cells were washed for three times with sterile PBS, split by ultra sonication. Finally, cell lysates were counted on the solid medium. The invasion rate was calculated according to the statistics of total microbe count / number of total cells.

2.8. Data analysis

The one-way analysis of variance (ANOVA) was used to analyze the data and the procedure was performed in Origin 8.0 software. All values from the replicates were expressed as mean ± standard deviation (SD). The significant different analyses were performed by Student's two-tailed *t*-test (Prism GraphPad, San Diego, CA). While *p* value less than 0.05, the difference was considered significant.

3. Results

3.1. Cloning of poIL-17B and poIL-17E

poIL-17B and *poIL-17E* were cloned, deposited in Genbank (*Sus scrofa IL-17B* gene, complete CDS, MH345768; *Sus scrofa IL-17E* gene, complete CDS, MH345769) and their biological characteristics analyses were performed (Fig. S1-4 in Supplemental file).

3.2. poIL-17B and poIL-17E expression in tissue and intestinal immune cells

In this study, 12 tissues of swine were selected, including small intestine, lung, liver, testis, testicle, muscle, brain, skin, spleen, trachea, kidney, stomach and heart corresponding to porcine digestive system, respiratory system, nervous system, exercise system, immune system and cardiovascular system. And the expression of poIL-17B and poIL-17E in these organs was detected. Relative expression of IL-17B was calculated by using spleen as control. The results of tissue distribution of IL-17B/E in porcine were shown in Fig. 1. The expression of poIL-17B in the kidney and lung was the highest. In addition, intestine had been detective high expression of poIL-17B and poIL-17E. In general, both poIL-17B and poIL-17E expression in the digestive system and respiratory system was higher than that of other tissues or systems.

According to the results of distribution of tissue (Fig. 1), the

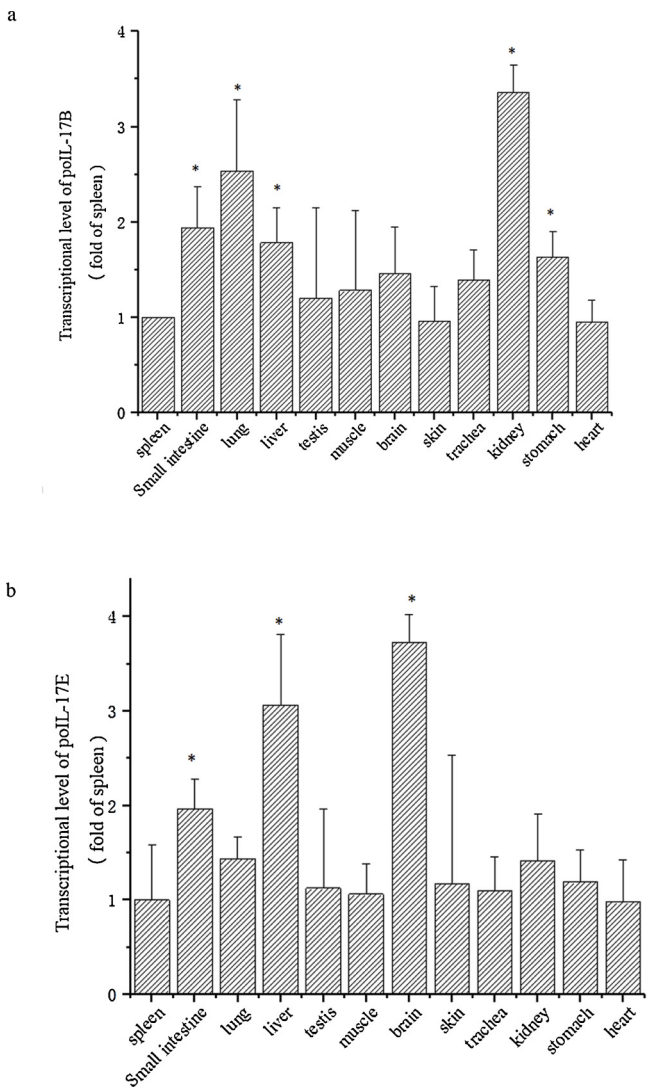

Fig. 1. The tissue distribution of poIL-17B (a) and poIL-17E (b) gene.
RNA samples were normalized by using the housekeeping gene, β-actin. Mean relative quantity of mRNA ± standard deviation (SD) was shown. Results were representative of three biological replicates. *$p < 0.01$ indicated significant difference vs. Spleen group.

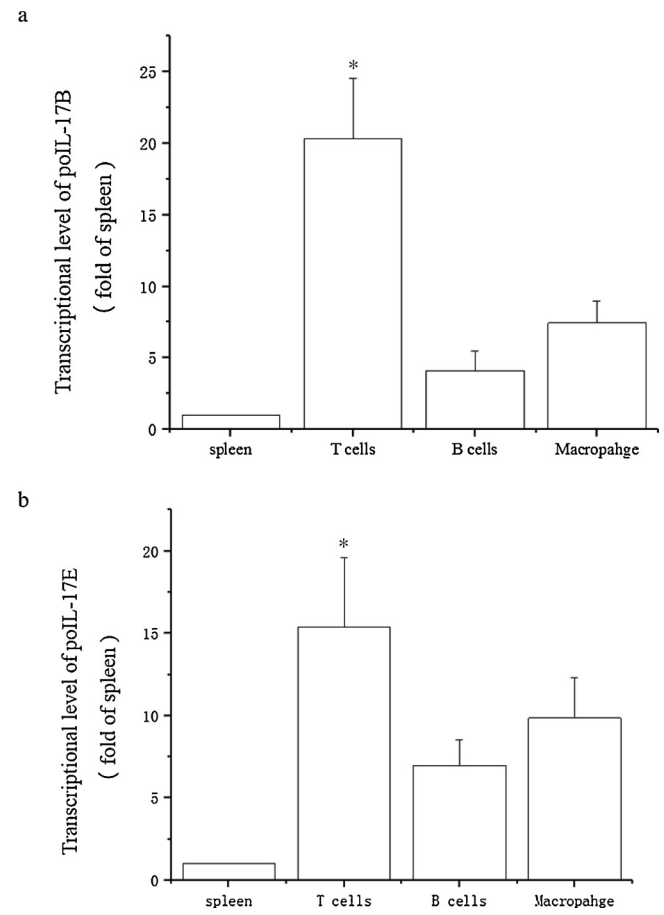

Fig. 2. The intestinal immune cells distribution of poIL-17B (a) and poIL-17E (b) gene.
RNA samples were normalized by using the housekeeping gene, β-actin. Mean relative quantity of mRNA ± standard deviation (SD) was shown. Results were representative of three biological replicates. *$p < 0.05$ indicated significant difference vs. B cells.

relatively high expression of IL-17B/E was intestine. Therefore, we examined the expression of poIL-17B/E in important intestinal immune cells, including T cells, B cells and macrophages. Our data showed that highest poIL-17B/E level was observed in T cells, moderately in macrophages and B cells (Fig. 2), which mirrored previous reports that IL-17 was primarily secreted by a distinct CD4 + T cell subset (Harrington et al., 2005).

3.3. Effect of poIL-17B and poIL-17E on cytokines and antibacterial peptide expression in IPEC J2 cells

Recombinant plasmids, named pcDNA3.1-poIL-17B and pcDNA3.1-poIL-17E, were constructed. Then they were transfected into IPEC-J2 cells, respectively. After culturing 48 h, relative expression of poIL-17B and poIL-17E were significantly enhanced ($p < 0.01$) (Fig. 3). The expression of cytokines (IL-1β, IL-8 and IL-10) and antibacterial peptides (PR-39, pBD-1, pBD-2 and pBD-3) were detected in IPEC-J2 cells, which were transfected by pcDNA3.1-poIL-17B and pcDNA3.1-poIL-17E. As shown in Fig. 4, poIL-17B did not affect the expression of IL-10 and pBD-2, while the expression of pBD-1 increased significantly (about 30.48 ± 0.70 fold, $p < 0.01$). The expression levels of IL-1β, IL-8, PR-39 and pBD-3 also increased in cells, which was transfected poIL-17B and poIL-17E respectively. In general, the expression of antibacterial peptides of poIL-17E group was higher than that of poIL-17B.

3.4. Expression poIL-17B and poIL-17E in IPEC J2 cells after co-culture with microorganisms

Pathogenic and probiotic microorganisms were used to stimulate IPEC-J2 cells for 6 h and 12 h, respectively. MOI = 1:1 and 10:1 (number of microbe: number of cells) were set. The expression of poIL-17B and poIL-17E in each strain were detected at 6 h (Fig. 5a) and 12 h (Fig. 5b) post stimulation. The levels of poIL-17B and poIL-17E expression were upregulated at different levels at 6 h post stimulation. However, the effects were more significant in strains *Pseudomonas aeruginosa, Saccharomyces cerevisiae* and *Candida albicans* than others ($p < 0.01$). When the MOI = 10, the expression level was more significant than MOI = 1, and the expression of poIL-17E was much higher than that of poIL-17B at MOI = 10. The general expression at 12 h post stimulation was higher than 6 h. The expression of poIL-17B/E was significantly ($p < 0.01$) increased after co-culture with strains *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Enterohemorrhagic Escherichia coli* and *Shigella sonnei*. In addition, the expression of poIL-17E was higher than that of poIL-17B.

When culturing with pathogenic microorganisms alone, the expression of poIL-17B and poIL-17E were both upregulated. However,

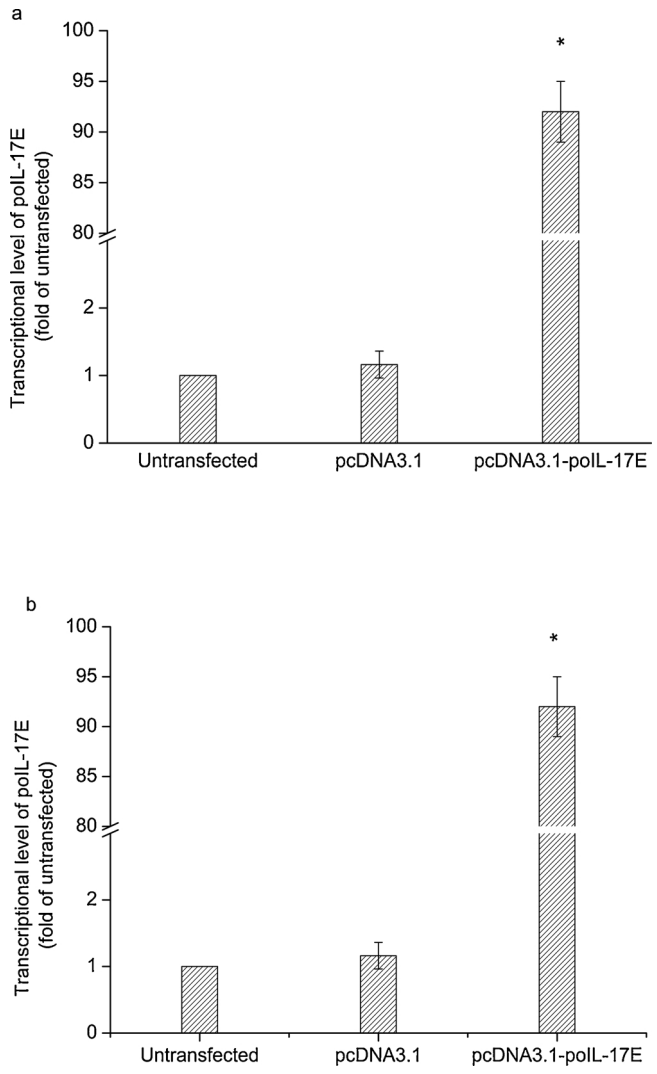

Fig. 3. Relative mRNA level of poIL-17B (a) and poIL-17E (b) in poIL-17B/E overexpressed IPEC J2 cells line.
pcDNA3.1-poIL-17B/E was transfected to IPEC J2 cells. The expression of poIL-17B/E in IPEC J2 cells line was detected. Results were representative of three biological replicates. * $p < 0.01$ indicated significant difference vs. untransfected cells groups.

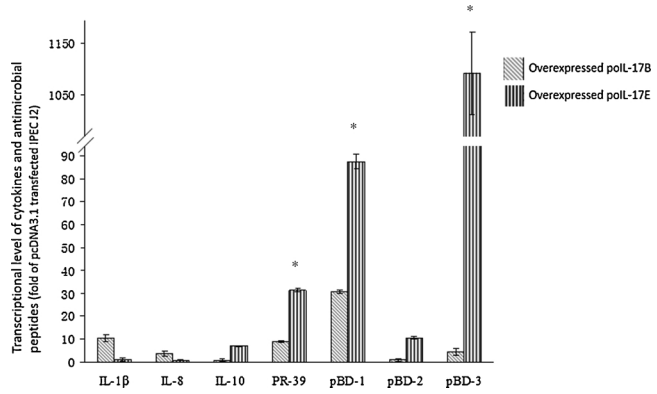

Fig. 4. Relative mRNA level of various cytokines and antimicrobial peptides in poIL-17B and poIL-17E overexpressed IPEC J2 cells line.
Cytokines and antimicrobial peptides mRNA levels were determined by qPCR using actin gene as housekeeping gene. Mean relative quantity of mRNA ± SD was shown. Results were representative of three biological replicates. *$p < 0.01$ indicated significant difference vs. poIL-17B group.

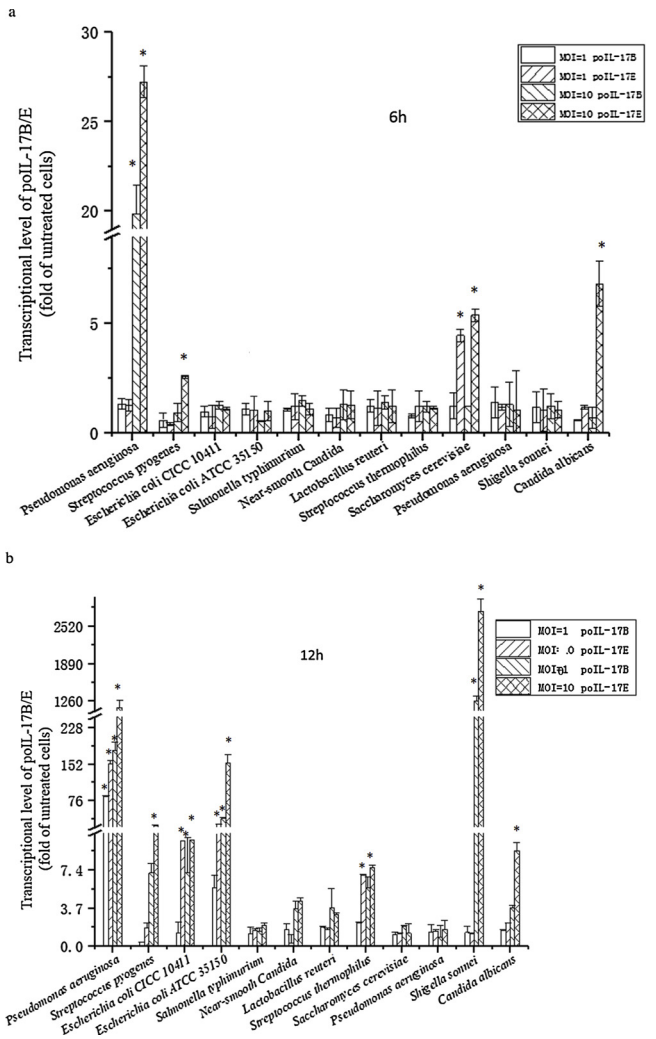

Fig. 5. Relative mRNA level of poIL-17B and poIL-17E after treatment with microorganism 6 h(a) and 12 h(b) respectively.
poIL-17B and poIL-17E mRNA levels were determined by qPCR using actin gene as housekeeping gene. In the infection model, the bacterial suspension were added to wells at 1 or 10 of MOI (multiplicity of infection). All the IPEC J2 cells were treated for 6 h (a) or 12 h (b), before the gene expression analysis. After the treatment, IPEC J2 cells was preserved for qPCR. Relative expression analysis of poIL-17B/E or β-actin were performed by the qPCR. Results were representative of three biological replicates. Mean relative quantity of mRNA ± SD was shown. *$p < 0.01$ indicated significant difference vs. untreated cells group.

after the addition of probiotics, the expression decreased significantly (Fig. 6, $p < 0.01$). Our data indicated that the probiotics could inhibit the expression of poIL-17B and poIL-17E.

3.5. Effect of poIL-17B and poIL-17E on invasion rate in IPEC J2 cells

The recombinant plasmids (pcDNA3.1-poIL-17B and pcDNA3.1-poIL-17E) were transfected into IPEC J2 cells and the transfected IPEC J2 cells were co-cultured with different pathogenic microorganisms. After culturing for 12 h, IPEC J2 cells were tested for infection with pathogenic microorganisms. The control group was IPEC J2 cells transfected with pcDNA3.1. As seen in Table 2, IPEC J2 cells transfected with pcDNA3.1-poIL-17B/pcDNA3.1-poIL-17E, were less susceptible to pathogenic microorganisms than the control group. This tabular data could speculate that poIL-17B and poIL-17E may be able to play a role in fighting against pathogenic microorganism's infection.

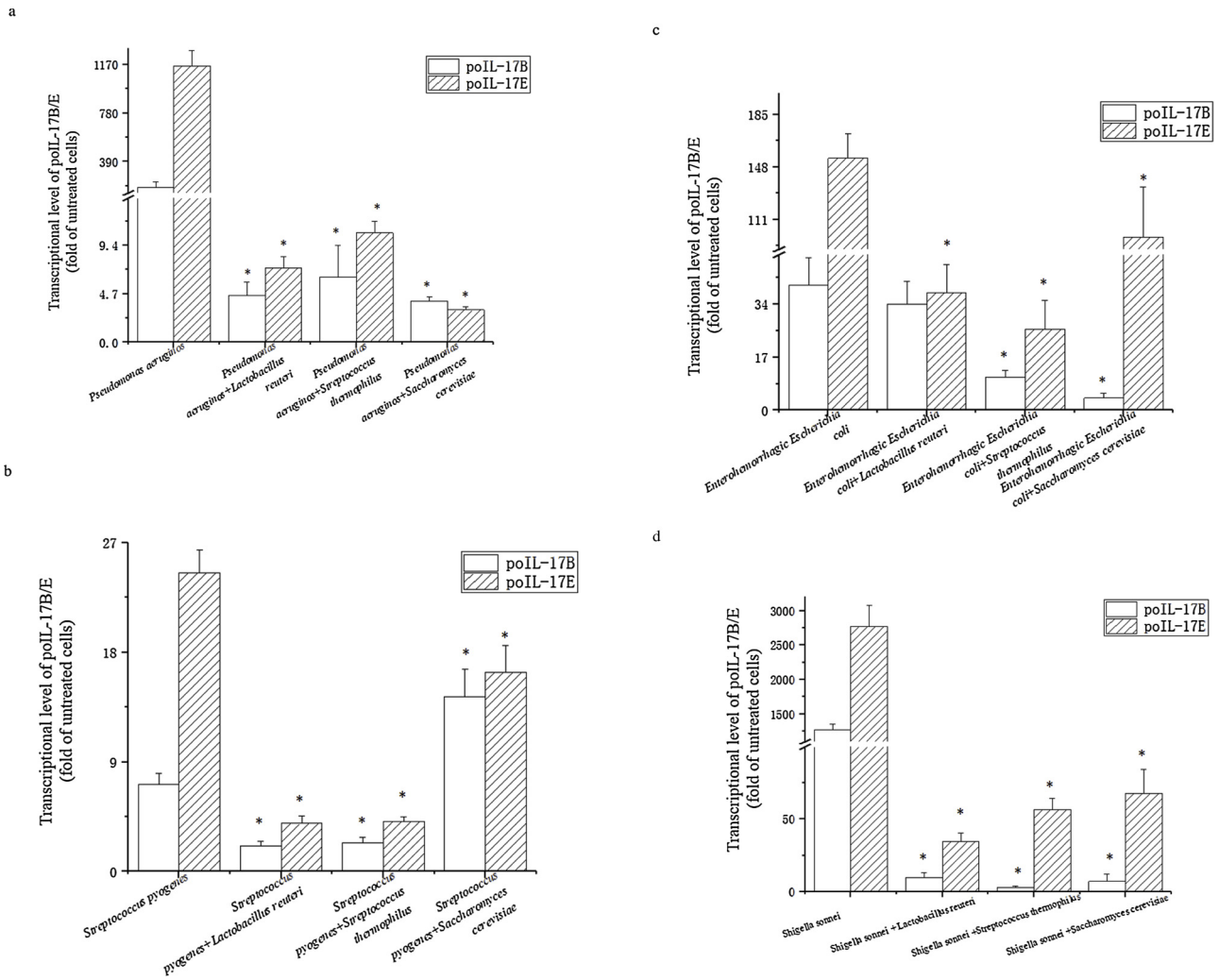

Fig. 6. Relative mRNA level of poIL-17B and poIL-17E after co-cultured with pathogenic microorganism (a: *Pseudomonas aeruginos,* b: *Streptococcus pyogenes*, c: *Enterohemorrhagic Escherichia coli*, d: *Shigella sonnei)* and probiotic.
poIL-17B and poIL-17E mRNA levels were determined by qPCR using actin gene as housekeeping gene. Mean relative quantity of mRNA ± SD was shown. In the microorganism treatments, the bacterial suspension were added to wells at 10 of MOI (multiplicity of infection) or pathogen togather with probiotics. IPEC J2 cells were treated for 12 h, before the gene expression analysis. After the treatment, IPEC J2 cells was preserved for qPCR. Relative expression analysis of poIL-17B/E or β-actin were performed by the RT-qPCR. Results were representative of three biological replicates. $*p < 0.01$ indicated significant difference vs. only co-culture pathogenic microorganism group.

Table 2
Effect of poIL-17B and IL-17E on invasion rate in IPEC J2 cells.

	pcDNA3.1 (CFU/cell)	pcDNA3.1-poIL-17B (CFU/cell)	pcDNA3.1-poIL-17E (CFU/cell)
Pseudomonas aeruginosa	$6.12 \pm 2.91 \times 10^3$	$9.21 \pm 2.31 \times 10^1$	$7.54 \pm 2.36 \times 10^2$
Shigella sonnei	$5.43 \pm 2.65 \times 10^3$	$4.12 \pm 1.18 \times 10^2$	$3.27 \pm 1.27 \times 10^3$
Enterohemorrhagic Escherichia coli	$4.43 \pm 1.32 \times 10^4$	$5.43 \pm 2.49 \times 10^3$	$8.83 \pm 1.04 \times 10^3$
Streptococcus pyogenes	$6.53 \pm 3.02 \times 10^3$	$6.24 \pm 3.60 \times 10^1$	$5.19 \pm 2.18 \times 10^2$

The recombinant plasmids (pcDNA3.1-poIL-17B and pcDNA3.1-poIL-17E) were transfected into IPEC J2 cells, and the transfected IPEC J2 cells were co-cultured with different pathogenic microorganisms. After culturing for 12 h, IPEC J2 cells were tested for infection with pathogenic microorganisms. The control group was IPEC J2 cells transfected with pcDNA3.1. Three clones have been analyzed for each cell type. Mean invasion rate ± standard deviation (SD) was shown.

4. Discussion

Intestinal infection is a common cause of diseases in animals. Intestinal immunity plays an important role in maintaining intestinal health. poIL-17B and poIL-17E are important in maintaining intestinal health and improving intestinal immunity. However, most of the related studies are mainly focused in mice and humans, and few about swine, which is one of the main economic animals. So far, porcine IL-17A has been cloned (Dirisala et al., 2013), but not porcine IL-17B/E. Because of unique intestinal immunity of human IL-17B/E, we set out to clone IL-17B/E in swine and analyze their immunomodulatory relationship with intestinal microbes.

Homology comparison revealed that poIL-17B and poIL-17E shared high similarity with the IL-17B and IL-17E protein in other domestic animal or human. However, phylogenetic tree (Fig S3f, S4f) illuminated that poIL-17B and poIL-17E was closed to domestic animal IL-17B and IL-17E, not human or murine. Thus, it was necessary to further investigated about the poIL-17B/E and pointed out more information about poIL-17B and poIL-17E.

The distribution of proteins in body tissues can reflect their function

to some extent. This study examined the expression of poIL-17B and poIL-17E in different tissues. According to data in Fig. 1, the expression of poIL-17B and poIL-17E were high in several mucosal tissues (including stomach, small intestine and lung), indicating that poIL-17B and poIL-17E were mainly related to intestinal immunity. However, the expression of IL-17B in mice was weak in lung, liver and skeletal muscle (Moseley et al., 2003). This suggested that there may be some difference in the function of this cytokines in mice and pigs. This study focused on explaining the role of poIL-17B and poIL-17E in gastrointestinal tissues. IPEC-J2 cells are a stable and reliable model of intestinal mucosal immune cells. Therefore, IPEC-J2 cells were used herein to study poIL-17B and poIL-17E. After transfection of recombinant plasmids pcDNA3.1-poIL-17B and pcDNA3.1-poIL-17E, the expression of poIL-17B and poIL-17E were indeed greatly increased ($p < 0.01$). When poIL-17E was over-expressed, the expression of antimicrobial peptides (PR-39, pBD-1, pBD-2 and pBD-3) was significantly increased ($p < 0.01$), and cytokine (IL-10) was moderately enhanced ($p < 0.01$). However, the expression of antimicrobial peptides induced by poIL-17B was lower than that of poIL-17E, while inflammatory cytokines (IL-1β and IL-8) were upregulated gently. It could be seen that poIL-17E and poIL-17B may own the function of inhibiting pathogenic infections by increasing the expression of antimicrobial peptides.

Meanwhile, cells were treated by several microbe with different biophysical properties, including probiotics (*Lactobacillus reuteri* DSM 20016, *Streptococcus thermophilus* ATCC 14485 and Saccharomyces cerevisiae CICC 1562), pathogen (*Pseudomonas aeruginosa* ATCC 27853, *Enterohemorrhagic Escherichia coli* ATCC 35150, *Streptococcus pyogenes* ATCC 19615 and *Shigella sonnei* CMCC 51592), conditional pathogens (*Enteropathogenic Escherichia coli* CICC 10411, *Salmonella Typhimurium* LT2, *Pseudomonas aeruginosa* PAO1; *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 20019). When MOI = 1 or 10, obvious upregulation of poIL-17B/E was observed after stimulation with several pathogens. Moreover, poIL-17E induction was higher than poIL-17B. After addition of probiotics (*Lactobacillus reuteri* DSM 20016, *Streptococcus thermophilus* ATCC 14485 and *Saccharomyces cerevisiae* CICC 1562), poIL-17E and poIL-17B cut down sharply. The infection of epithelial cells with pathogenic microorganisms significantly promoted the expression of poIL-17E and poIL-17B, especially poIL-17E. It is suggested that poIL-17B and poIL-17E could respond to infection of pathogen. In order to eliminate pathogens, immune cells were recruited (neutrophils, macrophages, etc.) which play an innate immune protective role in removing allogeneic agents) as an immune mechanism for the body to protect itself.

We also found that poIL-17B and poIL-17E could inhibit pathogen invasion in IPEC J2 cells (Table 2). Moreover, inhibitory effect of poIL-17B was stronger than poIL-17E. Reynolds demonstrated that IL-17B antagonized IL-25-mediated mucosal inflammation (Song et al., 2011). IL-17E was found to promote IL-6 production from colon epithelial cells, which was inhibited by IL-17B. Reynolds believed that IL-17B was an anti-inflammatory cytokine, while IL-17E was a pro-inflammatory cytokine in the IL-17 family (Reynolds et al., 2015). Our data mirrored pro-inflammatory function of poIL-17E since its expression level was upregulated extremely after pathogen infection (especially for *Pseudomonas aeruginosa* ATCC27853 and *Enterohemorrhagic Escherichia coli* ATCC 35150). So far, it has been reported that poIL-17E has the function of anti-parasitic infection, while the study of poIL-17B is rare. According to our results, poIL-17B and poIL-17E both could effectively inhibit pathogenic microorganism, while inhibitory capability of poIL-17B was stronger than that of poIL-17E. Therefore, we speculated that poIL-17B and poIL-17E both can be important members against intestinal infection in the porcine IL-17 family. The IL-17 family has been reported to play an important role in mucosal immunity (Abusleme and Moutsopoulos, 2017). So far, the mechanism for IL-17 in protecting against pathogens is believed as follows: 1)IL-17 promotes the production of antibacterial factors(Liang et al., 2006); 2) IL-17 induces the expression of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-8, and promotes the migration of neutrophils(Mantovani et al., 2011; Fossiez et al., 1996); 3)neutrophil recruitment is a major IL-17 function. Innate IL-17-producing cells are strategically positioned at barrier sites to sense injury and infection and rapidly recruit neutrophils for initial containment of any insult(Onishi and Gaffen, 2010); 4)IL-17 maintains the barrier and maintains intestinal contents and symbiotic organisms by modulating tight junction proteins that connect and stabilize epithelial cell junctions (Karp et al., 2010). In our study, the expression level of antimicrobial peptide (PR-39, pBD-1, pBD-2 and pBD-3) was indeed significantly upregulated after overexpression of IL-17B/E in IPEC-J2, the inflammatory factors IL-1β and IL-8 increased in IL-17B-overexpressed cells. Our results were consistent with this mechanism.

However, IL-17 could clear the pathogenic microorganism, *Candida albicans,* by recruiting neutrophils and inducing antibacterial agents (Conti et al., 2009). People who developed damage during Th17 cell development did show susceptibility to selected bacterial and fungal infections, and patients with defective Th17 cell differentiation were susceptible to *S. aureus, S. pneumoniae* and *Haemophilus* influenzae infection (Milner et al., 2008). In addition, studies had shown that *Staphylococcus aureus, Bifidobacterium adolescentis* and *Enterotoxin-producing Bacteroide*s (ETBF), had the activity of inducing Th17 cells and the composition of intestinal microbes affected the Th17 immune responses (Tan et al., 2016; Atarashi et al., 2015). These findings indicated that microbe and Th17 cells could interact each other. According to these reports along with our findings, we speculated, to some degree, that swine intestinal epithelial cells up-regulate poIL-17B/E in the face of external microbial infections. Firstly, poIL-17B/E can promote the formation of a protective immune response, and simultaneously up-regulate the expression of antimicrobial peptides, which may also be the body's own immune defense mechanism. Secondly, our investigation data showed poIL-17B/E overexpression could inhibit the invasion of pathogenic microorganisms. When probiotics were present, they helped to smooth out these inflammatory medium by alleviating poIL-17B/E levels. This is because excessive inflammation, on the one hand, inhibits pathogenic microbes; on the other hand, it can be harmful to the gut itself. Probiotics have the function of protecting the intestinal mucosa, which make the immune response induced by the pathogenic microorganisms more peaceful. Therefore, we believed that poIL-17B and poIL-17E played an important role in intestinal anti-infection, and improved the efficiency against intestinal pathogenic microorganisms.

In our study, we investigated the effects of microorganisms on IL-17B/E. In fact, these cytokines have been a modest activator of in vitro signaling, regardless of the cell system analyzed (Veldhoen, 2017). IL-17 was capable of interacting with a variety of mediators such as TNF-α and FGF2, and activating different signaling pathways (Song et al., 2015; Chiricozzi et al., 2011). The molecular basis for this synergy is not fully understood and may involve multiple mechanisms. In addition, IL-17E and IL-17B share common receptor IL-17RB (Huang et al., 2014). IL-17B competes with IL-17E for binding to IL-17RB. IL-17B inhibits the pro-inflammatory function of IL-17E (Reynolds et al., 2015). Our understanding of these nuances is still in its infancy, particularly in the areas of tuning of IL-17B/E responses by diverse cytokines and microbial stimulation. These areas need further exploration.

In conclusion, we had successfully cloned and expressed poIL-17E and poIL-17B. In addition, biological characteristics of two proteins were analyzed. Our data showed that poIL-17E and poIL-17B could significantly promote the expression of antibacterial peptides. It suggested that they played a role in intestinal anti-infectious immunity. The expression of poIL-17E and poIL-17B was significantly up-regulated after infection with pathogen (*Pseudomonas aeruginosa* ATCC27853, *Enterohemorrhagic Escherichia coli* ATCC 35150 and *Candida albicans* ATCC 10231). When probiotics were added, the expression was down-regulated, and poIL-17E were greater than that of poIL-17B. Meanwhile, poIL-17E or poIL-17B both could inhibit the invasion of pathogens, and the effect of poIL-17B was greater than that of poIL-17E. Therefore, we believed that, to some extent, both poIL-17E and poIL-

17B play a role in controlling the degree of infection in the intestinal anti-infective immunity. This study provides a theoretical basis for the prevention of intestinal diseases in pigs.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.10.011.

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