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Oral administration of *Lactobacillus rhamnosus* GG to newborn piglets augments gut barrier function in pre-weaning piglets*#

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Abstract: To understand the effects of *Lactobacillus rhamnosus* GG (ATCC 53103) on intestinal barrier function in pre-weaning piglets under normal conditions, twenty-four newborn littermate piglets were randomly divided into two groups. Piglets in the control group were orally administered with 2 mL 0.1 g/mL sterilized skim milk while the treatment group was administered the same volume of sterilized skim milk with the addition of viable *L. rhamnosus* at the 1st, 3rd, and 5th days after birth. The feeding trial was conducted for 25 d. Results showed that piglets in the *L. rhamnosus* group exhibited increased weaning weight and average daily weight gain, whereas diarrhea incidence was decreased. The bacterial abundance and composition of cecal contents, especially Firmicutes, Bacteroidetes, and Fusobacteria, were altered by probiotic treatment. In addition, *L. rhamnosus* increased the jejunal permeability and promoted the immunologic barrier through regulating antimicrobial peptides, cytokines, and chemokines via Toll-like receptors. Our findings indicate that oral administration of *L. rhamnosus* GG to newborn piglets is beneficial for intestinal health of pre-weaning piglets by improving the biological, physical, and immunologic barriers of intestinal mucosa.

Key words: *Lactobacillus rhamnosus*; Gut microbiota; Intestinal physical barrier; Intestinal immunological barrier; Piglet
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1 Introduction

Diarrhea, an important cause of morbidity and mortality in livestock, is one of the most common diseases of suckling piglets worldwide (Toledo et al., 2012). In October 2010, the severe outbreak of porcine epizootic diarrhea in southern China caused the death of more than 1 000 000 piglets (Sun et al., 2012). Diarrheal disease was also reported in European countries (Hanke et al., 2015; Theuns et al., 2015) and spread rapidly to approximately 50% of the US swine breeding herds from July 2013 to July 2014 (Goede and Morrison, 2015). To fight against diarrhea, antibiotics have been applied widely in neonatal piglets to enhance gut function and prevent diarrhea (Hermann-Bank et al., 2015; Ngamwongsatit et al., 2016). However, the promiscuous use of antibiotics could influence the balance of gut bacteria and result

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in the emergence and spread of resistant bacteria (Kemper, 2008). Since bacterial colonization of the mammalian gastrointestinal tract begins at birth (Hermann-Bank et al., 2015), it is necessary to establish a healthy intestinal microbiota of newborn suckling piglets. Gut microbiota constitutes the intestinal biological barrier (Natividad and Verdu, 2013; Kelly et al., 2015), prevents pathogen colonization, and converts indigestible substances into digestible components that benefit the host and help in the maturation of the gastrointestinal tract as well as of the immune system (Guarner and Malagelada, 2003; Bauer et al., 2006; Ley et al., 2008; Koenig et al., 2011). As well as the biological barrier, the physical barrier and immune mediators are also important in defending against pathogens (McCracken and Lorenz, 2001). In the presence of an intact epithelial cell layer, the paracellular pathway between cells must be sealed. This function is achieved by a physical barrier, especially tight junctions, which are multi-protein complexes to limit solute flux along the paracellular pathway. The tight junction is, therefore, the rate-limiting step in transepithelial transport and the principal determinant of mucosal permeability (Turner, 2009). Additionally, the cytokines and chemokines secreted by enterocytes and the antimicrobial peptides secreted by Paneth cells could regulate bacterial interactions with the mucosal surface, so as to maintain the intestinal immune homeostasis (Rescigno, 2011).

Probiotics are nonpathogenic bacteria that exert a beneficial influence on host health, physiology, or both (Rajput et al., 2013), and further improve intestinal structure, aid in the development of immunity to defend against pathogens, and subsequently improve growth performance (Lei et al., 2013). In animal production, several selected probiotics have been applied to improve animal health and performance, such as *Lactobacillus*, *Streptococcus*, *Saccharomyces*, *Aspergillus*, and *Bacillus* species (Tannock, 2001). As a prominent probiotic member, *Lactobacillus* could augment intestinal health by modulating the gut microbiota of mice with metabolic syndrome (Wang et al., 2015), attenuating the negative effects of alcohol on mouse tight junction expression (Chen et al., 2016), and activating the gut mucosal immune system of mice (Galdeano and Perdigón, 2006). Although there are some reports on probiotics' effects on pig intestinal barrier functions (Zhang et al., 2010; Li et al., 2012; Deng et al., 2013; Hou et al., 2015), few studies have administered *Lactobacillus rhamnosus* to newborn piglets in the first few days after birth to determine its protective effects on intestinal functions without challenging with pathogens. Therefore, this study was conducted to determine the effects of orally administered *L. rhamnosus* on the intestinal barrier function in pre-weaning piglets under normal conditions.

2 Materials and methods

2.1 Bacterial preparation

The *L. rhamnosus* GG (ATCC 53103) in freeze-dried powder form was purchased from the China Center of Industrial Culture Collection (CICC), and cultured in de Man-Rogosa-Sharpe (MRS) medium (Oxoid, Basingstoke, UK) in anaerobic condition at 37 °C until reaching the logarithmic phase. Then the bacterial strain was separated by centrifugation (15 min at 3000*g*) and washed twice with sterile phosphate-buffered saline (pH 7.4). Thereafter, the bacteria were re-suspended in 0.1 g/mL sterile skim milk made from cows' milk powder to prepare the required concentration (5×10^8 CFU/mL).

2.2 Animals and treatments

Six sows (Duroc×Landrace×Yorkshire) were participants in this experiment. Four newborn piglets with similar initial weights were selected from each sow's offspring. The twenty-four newborn piglets were then randomly divided into two groups. Each group had three litters with four pigs per litter (half male and half female). The basal diet was supplemented with minerals and vitamins to meet or exceed the requirements for pigs (National Research Council, 1998). All pigs were fed ad libitum. The experiment was approved by and performed in accordance with the guidelines of the Zhejiang University Animal Care and Use Committee, Hangzhou, China. Piglets in the control group were orally administered with 2 mL of sterile skim milk, while the treatment group (*L. rhamnosus* group) received the same volume of sterile skim milk suspended with viable *L. rhamnosus* (5×10^8 CFU/mL) by gavage at the 1st, 3rd, and 5th days after birth. From Day 12, piglets were provided with free access to water and a supplemented pre-starter feed. Body weights

were recorded at the beginning and the weaning day (the 25th day) in this experiment. Diarrhea was observed every day. Composition and nutrient levels in the diets of sows are listed in Tables S1 and S2, respectively. Composition and nutrient levels in the diets of the pre-starter feed were listed in Tables S3 and S4, respectively. No antibiotic was added throughout the trial. The schematic flow diagram of the study design can be found in Fig. S1.

2.3 Sample collection

On Day 25, six piglets (half male and half female) were randomly picked from each group to collect intestine samples. Briefly, ketamine (11 mg/kg) and xylazine (1.5 mg/kg) were injected to minimize stress. Thereafter, chemical euthanasia was performed using an overdose of intravenous pentobarbital via a catheterized ear vein (Li et al., 2012). The mid-jejunum segments were carefully dissected and rinsed with sterilized saline. Mucosae were gently scraped off and cecal contents were carefully squeezed out, then placed in liquid nitrogen immediately, and stored at −80 °C till further analysis.

2.4 ELISA

The mucosa samples were diluted 1:2 (v/v) in sterile saline solution and homogenized with a glass homogenizer. Then the homogenates were centrifuged at 3000*g* for 15 min at 4 °C and supernatants were collected for the measurement of the concentrations of interleukin-6 (IL-6), IL-8, IL-10, IL-12, transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) by porcine enzyme-linked immunosorbent assay kit (ELISA kit; R&D Systems, Inc., Minneapolis, USA) as per the manufacturer's instructions. Data are presented as pg/g wet weight (Li et al., 2012).

2.5 Diamine oxidase assay

The mucosa samples were homogenized with ice-cold physiologic saline (1:10, w/v) and centrifuged at 2000*g* for 10 min (Centrifuge, Eppendorf, Germany). Supernatant was collected for determination of the diamine oxidase (DAO) activity. The kit for DAO was obtained from Nanjing Bioengineering Institute, Nanjing, China, and the DAO value was determined by spectrophotometry according to the manufacturer's instructions.

2.6 RNA extraction and qRT-PCR

Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) were performed according to Wang et al. (2017). The primer sequences used for qRT-PCR are listed in Table 1. The $2^{-\Delta\Delta C_T}$ method was used to estimate mRNA abundance. Relative gene expression levels were normalized by the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

2.7 DNA extraction

Genomic DNA was extracted from every cecal content sample using a TIANamp Stool DNA kit according to the manufacturer's protocols (Tiangen Biotech, Beijing, China).

2.8 454 pyrosequencing

The 454 pyrosequencing was performed according to Zhang et al. (2014). Amplification of the bacterial V3 region of the 16S rRNA gene was conducted using a primer set (Casserly and Erijman, 2003), and the 5' terminus of each forward and reverse primer contained an 8-bp barcode sequence to tag specific samples (Table 2).

2.9 Statistical analysis

Data are presented as means with their standard deviations (SDs). They were analyzed with SPSS 16.0 for Windows, using independent samples *t*-test. Differences were considered statistically significant at $P<0.05$ or $P<0.01$. Different superscripts denote significant difference between groups.

3 Results

3.1 *L. rhamnosus* improved growth performance

Compared with the control group, *L. rhamnosus* significantly increased the final body weight and daily weight gain, while decreasing the diarrhea rate from 6.41% to 4.44% (Table 3).

3.2 *L. rhamnosus* regulated bacterial diversity and composition in cecal contents

The phylogenetic differences within the intestinal microbiota were assessed by principal component analysis (PCoA) (Fig. 1). *L. rhamnosus* treatment

Table 1 Gene names, primer sequences, and product sizes

Gene symbol	Gene name	GenBank accession No.	Primer sequence	Product size (bp)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001206359.1	F: 5'-ATGGTGAAGGTCGGAGTGAAC-3' R: 5'-CTCGCTCCTGGAAGATGGT-3'	235
MUC2	Mucin 2	XM_002347185.2	F: 5'-ACGACTTTGACGGACACTGCT-3' R: 5'-AGGGGACGTTCTCGGTGAT-3'	99
pBD-1	Porcine β-defensin-1	NM_213838.1	F: 5'-CGCCTCCTCCTTGTATTCCTC-3' R: 5'-GGTGCCGATCTGTTTCATCTT-3'	144
PMAP-37	Porcine myeloid antimicrobial peptide 37	NM_213863.1	F: 5'-CACCTGCAATGAGGGTGTCA-3' R: 5'-GTCGCAACCGTGGTCTTCG-3'	68
LYZ	Lysozyme C-2 precursor	NM_214392	F: 5'-CCGCTACTGGTGTAATGATGG-3' R: 5'-ATGCTTTAACGCCTAGTGGAT-3'	143
OCLN	Occludin	NM_001163647	F: 5'-AATGCTTTCTCAGCCAGCGTAT-3' R: 5'-GCAAGCGTGGAGGCAACA-3'	153
ZO-1	Zonula occludens-1	XM_003121673.1	F: 5'-GCCTCCTGAGTTTGATAGTGG-3' R: 5'-CTCGGCAGACCTTGAAATAGA-3'	287
MCP-1	Monocyte chemoattractant protein-1	NM_214214.1	F: 5'-AGAAGAGTCACCAGCAGCAAG-3' R: 5'-TAGGGCAAGTTAGAAGGAAATG-3'	206
TNF-α	Tumor necrosis factor-α	NM_214022.1	F: 5'-CATCGCCGTCTCCTACCA-3' R: 5'-CCCAGATTCAGCAAAGTCCA-3'	199
IFN-γ	Interferon-γ	NM_213948.1	F: 5'-GAGCCAAATTGTCTCCTTCTAC-3' R: 5'-CGAAGTCATTCAGTTTCCCAG-3'	140
TGF-β1	Transforming growth factor-β1	NM_214015.1	F: 5'-GGACCTTATCCTGAATGCCTT-3' R: 5'-TAGGTTACCACTGAGCCACAAT-3'	133
IL-8	Interleukin-8	NM_213867.1	F: 5'-ATGCCAGTGCATAAATACGC-3' R: 5'-TTGGGAGCCACGGAGAAT-3'	251
TLR2	Toll-like receptor 2	NM_213761.1	F: 5'-GGTCCGATGCTGGTCTTTAT-3' R: 5'-GCAAGTCACCCTTTATGTTATTCA-3'	83
TLR9	Toll-like receptor 9	NM_213958.1	F: 5'-CCCACGACAGCCGAATAG-3' R: 5'-GGAACAGGGAGCAGAGCA-3'	122
TLR6	Toll-like receptor 6	NM_213760.1	F: 5'-TCTGCTCAAGGACTTCCGTGT-3' R: 5'-CAGCCCAGTGACTCCGATG-3'	79
TLR8	Toll-like receptor 8	NM_214187.1	F: 5'-GGATACCATTGCGGCGATAA-3' R: 5'-CCAGGGCAGCCAACATAACT-3'	71

F: forward; R: reverse

had a distinct microbiota composition that clustered separately from control diet-fed piglets. The α-diversity (richness and evenness) of the communities was measured by Shannon, chao1, PD_whole_tree, observed_species, and goods_coverage's indices (Table 4). However, no significant differences were observed between the two groups. To assess specific changes in the gut microbiota, we compared the relative abundance of the phylum identified from sequencing (Fig. 2). Results showed that Firmicutes, Bacteroidetes, and Fusobacteria phyla were dominant in the control group, comprising 33.56%, 35.89%, and 15.20% of the sequences, respectively; however, in *L. rhamnosus* group, Firmicutes percentage was dominant and the Bacteroidetes proportion was decreased. Noticeably, Fusobacteria almost disappeared in the *L. rhamnosus* group (Fig. 2).

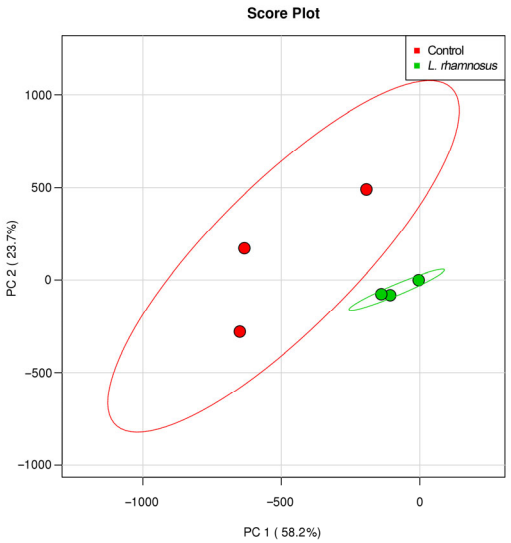

Fig. 1 Principal component analysis for the piglets' gut microbiota

Table 2 Barcode sequences and primer sequences

Group	Primer	Barcode sequence	Primer sequence (5'→3')
Control	V3-60F	AGATACTG	AGATACTGCCTACGGGAGGCAGCAG
	V3-60R		AGATACTGATTACCGCGGCTGCT
	V3-61F	AGAGTATG	AGAGTATGCCTACGGGAGGCAGCAG
	V3-61R		AGAGTATGATTACCGCGGCTGCT
	V3-63F	AGAGTGAT	AGAGTGATCCTACGGGAGGCAGCAG
	V3-63R		AGAGTGATATTACCGCGGCTGCT
L. rhamnosus	V3-55F	AGATATGT	AGATATAGCCTACGGGAGGCAGCAG
	V3-55R		AGATATAGATTACCGCGGCTGCT
	V3-58F	AGATAGAT	AGATAGTGCCTACGGGAGGCAGCAG
	V3-58R		AGATAGTGATTACCGCGGCTGCT
	V3-62F	AGATACAT	AGAGTAGTCCTACGGGAGGCAGCAG
	V3-62R		AGAGTAGTATTACCGCGGCTGCT

Table 3 Body weight gain and diarrhea rate of suckling piglets

Group	Body weight at birth (kg)	Final body weight (kg)	Daily weight gain (kg)	Diarrhea rate (%)
Control	1.43±0.03	6.67±0.13^{b}	0.22±0.01^{b}	6.41±0.72^{a}
L. rhamnosus	1.38±0.04	7.62±0.13^{a}	0.25±0.01^{a}	4.44±0.34^{b}

The data followed by the different small letters in the same column are significantly different (P<0.05). Diarrhea rate (%)=(number of piglets with diarrhea in seven days/total number of piglets in the group)×100%. Data are expressed as mean±SD (n=12)

Table 4 Changes in α-diversity of gut microbiota communities

Group	Shannon	Chao1	PD_whole_tree	Observed_species	Goods_coverage
Control	5.15±0.71	251.13±105.56	11.02±3.05	178.67±62.74	0.98±0.00
L. rhamnosus	5.17±1.18	195.65±149.13	9.36±2.17	127.00±66.47	0.97±0.02

Data are expressed as mean±SD (n=3)

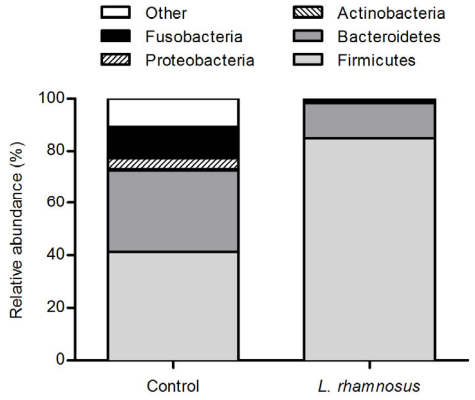

Fig. 2 Composition of gut microbiota at phylum level
The abundance is presented in terms of a percentage of the total effective bacterial sequences in the sample, which were classified using the RDP Classifier at a confidence threshold of 50%

3.3 *L. rhamnosus* regulated mRNA expression of genes related to physical barrier function

The results of expression of genes related to tight junctions in jejunal mucosa showed that the transcript level of occludin (*OCLN*) was significantly down-regulated by 48.12% with *L. rhamnosus* treatment, while no significant difference was found in the zonula occludens-1 (*ZO-1*) mRNA expression between the two groups (Fig. 3). *L. rhamnosus* increased mucin-2 (*MUC-2*) gene expression slightly (Fig. 3), but DAO activity was significantly decreased (Fig. 4).

3.4 *L. rhamnosus* improved intestinal immune barrier function

Oral administration of *L. rhamnosus* significantly up-regulated the mRNA levels of porcine β-defensin-1 (*pBD-1*) and porcine myeloid antimicrobial peptide 37 (*PMAP-37*), but decreased the expression of lysozyme C-2 precursor (*LYZ*) in jejunal mucosa of the suckling piglets (Fig. 5a). Results in Fig. 5b illustrate that *L. rhamnosus* markedly up-regulated the mRNA levels of both pro-inflammatory cytokines (*TNF-α*, *IFN-γ*) and the anti-inflammatory cytokine (*TGF-β1*). In the *L. rhamnosus* treatment group, chemokine *MCP-1* mRNA expression was almost

quadrupled compared with that in the control group (1.00 vs. 3.88). Despite this, most of the pro-inflammatory cytokine concentrations in jejunal mucosa were significantly decreased in the *L. rhamnosus* group, including IL-1β, IL-6, IL-12, IL-8, and TNF-α, whereas the IFN-γ concentration was increased (Table 5), which was in line with the qRT-PCR result (Fig. 5b). Additionally, production of anti-inflammatory cytokines, such as IL-10 and TGF-β1, was markedly increased with oral administration of *L. rhamnosus* (Table 5).

3.5 *L. rhamnosus* regulated transcript levels of key genes involved in innate immune signaling pathways in jejunal mucosa

In the *L. rhamnosus* group, Toll-like receptor 2 (*TLR2*), *TLR9*, and myeloid differentiation-2 (*MD-2*) mRNA expression levels were dramatically up-regulated. However, no significant difference was observed in *TLR6* or *TLR8* expression between the two groups (Fig. 5c). To determine whether *L. rhamnosus* played a role in innate immune signaling pathways, we detected mRNA expression of TGF-β activated kinase 1 (*TAK1*), TNF receptor associated factor 6 (*TRAF6*), c-Jun N-terminal kinase (*JNK*), *p38* and peroxisome proliferator activated receptor γ (*PPARγ*) (Fig. 5d), but only *TRAF6* expression was significantly increased by *L. rhamnosus*.

4 Discussion

Immediately after birth, the gastrointestinal tract of neonates is involved in a process of microbiota colonization and succession (Mackie et al., 1999; Gaskins et al., 2008). Increasing evidence has suggested that early exposure with desirable microbiota could alter the pattern of microbial succession as well as immunological maturation (Rakoff-Nahoum and Medzhitov, 2008; Hou et al., 2015). The use of probiotics in piglet nutrition has been increasingly discussed in recent years (Taras et al., 2006; Ayala et al.,

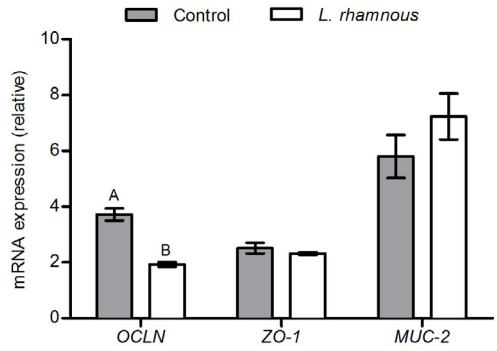

Fig. 3 Relative expression levels of *OCLN*, *ZO-1*, and *MUC-2* in jejunal mucosa of piglets
The different capital letters on the bar mean that the values are very significantly different (P<0.01). Data are expressed as mean±SD (n=6)

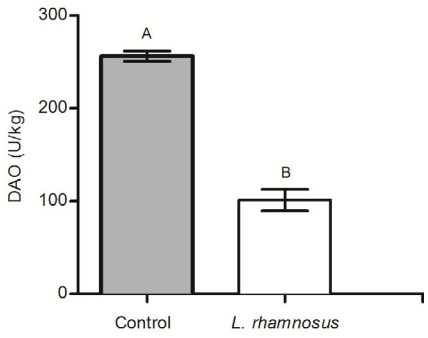

Fig. 4 DAO activity in jejunal mucosa of piglets
The different capital letters on the bar mean that the values are very significantly different (P<0.01). Data are expressed as mean±SD (n=6)

Table 5 Production of cytokines and chemotactic factor in jejunal mucosa of suckling piglets

Group	IL-1β (pg/g)	IL-6 (pg/g)	IL-12 (pg/g)	TNF-α (pg/g)
Control	3244.33±90.22^{A}	164.43±9.30^{A}	263.24±16.01^{A}	96.95±11.76^{A}
L. rhamnosus	2133.55±46.21^{B}	89.78±5.22^{B}	161.14±4.39^{B}	31.62±3.63^{B}
Group	IFN-γ (pg/g)	IL-8 (pg/g)	IL-10 (pg/g)	TGF-β1 (pg/g)
Control	4721.51±268.08^{B}	25 102.68±468.97^{A}	199.16±14.92^{B}	149.19±3.78^{B}
L. rhamnosus	6733.47±254.09^{A}	14 776.40±512.04^{B}	242.55±9.01^{A}	200.97±7.44^{A}

The data followed by different capital letters in the same column are very significantly different (P<0.01). Data are expressed as mean±SD (n=6)

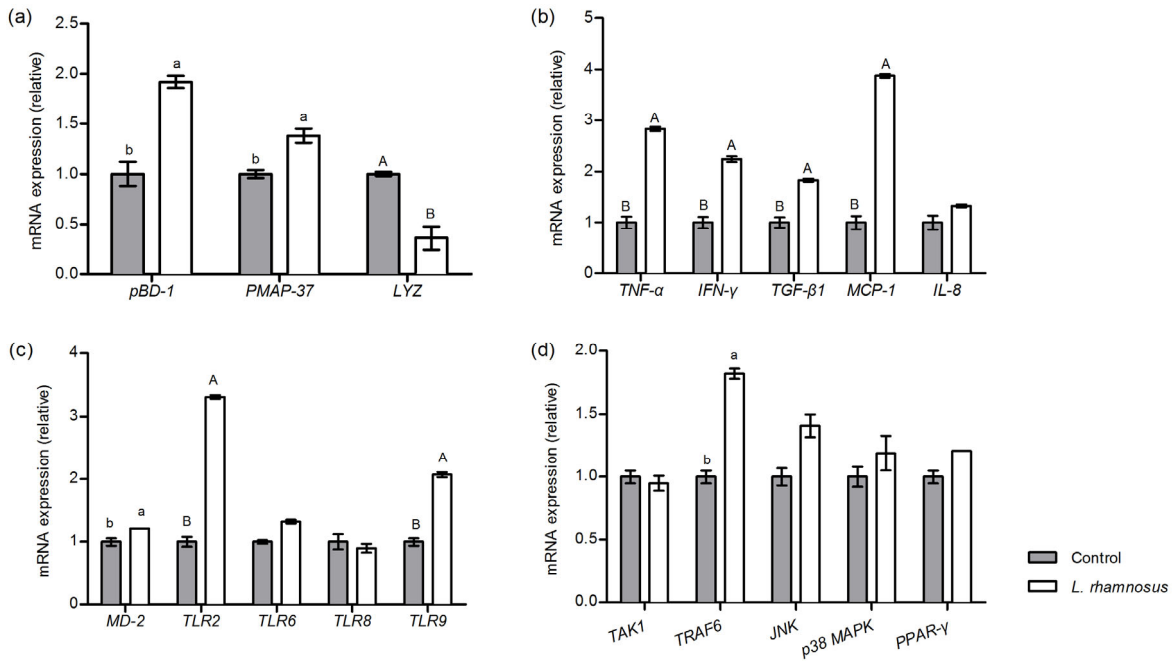

Fig. 5 Effect of *L. rhamnosus* on the immune barrier function of piglets

(a) Relative expression of *pBD-1*, *PMAP-37*, and *LYZ* in jejunal mucosa; (b) Relative expression of cytokine and chemotactic factor genes in jejunal mucosa; (c) Relative expression of *TLR* genes in jejunal mucosa; (d) Relative expression of innate immune signaling relative genes in jejunal mucosa. The different small letters or different capital letters on the bar mean that the values are significantly different (P<0.05) or very significantly different (P<0.01), respectively. Data are expressed as mean±SD (n=6)

2015; Patil et al., 2015). Many studies have found beneficial effects of probiotics supplementation on the growth performance and gut health of suckling piglets (Shim et al., 2005; Zeyner and Boldt, 2006; Hayakawa et al., 2016). However, little is known about the piglets' intestinal functions following *L. rhamnosus* treatment in the first few days after birth under normal conditions. Thus, the objective of this experiment is to determine whether *L. rhamnosus* administration to newborn piglets in the first few days after birth can enhance the intestinal barrier functions, including the intestinal immune function, intestinal permeability, and gut microbial composition of pre-weaning piglets.

As is known, *L. rhamnosus* can improve the daily weight gain of piglets (Bocourt et al., 2004a, 2004b) and ameliorate diarrhea (Guarino et al., 2009). In the present study, *L. rhamnosus* also effectively increased the growth performance of pre-weaning piglets and alleviated the diarrhea, which implied that *L. rhamnosus* might improve the function of the intestinal barrier to defend against enteric pathogens (Nalle and Turner, 2015). Recently, accumulating evidence has suggested that gut microbiota plays a key role in gut health (Gareau et al., 2010; Flint et al., 2012). Intestinal bacterial communities are comprised of more than 1000 different species (Lozupone et al., 2012) and constitute an exceptionally diverse microbial ecosystem, which is critical for numerous physiologic processes (Stappenbeck et al., 2002; Hooper, 2004; Mazmanian et al., 2005). Genera of the phylum Firmicutes, which comprise the dominant microflora in the gut (Eckburg et al., 2005), including *Bacillus*, *Enterococcus*, *Lactobacillus*, and *Lactococcus*, play an important role in human and animal health (Haakensen et al., 2008). Although *Bacteroidetes* species also boost animal growth and health (Salyers, 1984), enterotoxigenic *B. fragilis*, a human colonic commensal, has been demonstrated to increase colonic thickness, inflammation and visible colonic tumors (Wu et al., 2009). Zhu et al. (2014) also found that *Bacteroidetes* species were more abundant in rats with colorectal cancer. *Fusobacteria* species have long been recognized in a wide spectrum of human

infections (Klinge et al., 2002; Gmür et al., 2006; Allen-Vercoe et al., 2011). In the analysis of bacterial composition in cecal contents in this study, we observed that at the phylum level, the most remarkable result was the increase in Firmicutes and the decrease in Bacteroidetes as well as Fusobacteria with *L. rhamnosus* treatment. Similarly, the study of Angelakis and Raoult (2010) also noticed that *Lactobacillus* inoculation improved the weight gain via increasing the Firmicutes/Bacteroidetes ratio in the gut flora of newborn chicks and ducks. Hence, we speculate that *L. rhamnosus* could exert beneficial effects on piglets' health via modulating the bacterial composition, especially the abundance of Firmicutes, Bacteroidetes, and Fusobacteria.

Microflora colonization can also be influenced by intestinal MUC-2, which is a major gel-forming mucin and is in direct contact with gut bacteria (Zhang et al., 2015). It was reported that dietary *Lactobacillus* supplementation increased MUC-2 concentration (Mao et al., 2016). Here, *MUC-2* expression was also up-regulated by *L. rhamnosus*, but not to a statistically significant extent. DAO is an intracellular enzyme in the mucosal villous epithelial cells of humans and all mammals; the highest activity is detected in the jejunum and ileum (Meng et al., 2016). Following damage, necrosis, and exfoliation of the intestinal mucosal cells, DAO translocates into blood, which implies the destruction of the intestinal mucosal barrier and changes in intestinal permeability (Swank and Deitch, 1996; Sun et al., 2011). Compared with the control group, oral administration of *L. rhamnosus* significantly decreased DAO in jejunal mucosa. In addition, unlike the results in other papers (Mennigen et al., 2009; Liu et al., 2013), the tight junction *OCLN* in the present study was down-regulated when exposed to *L. rhamnosus*. As far as we are concerned, when the gut epithelium is damaged, the expression of tight junction proteins can also be increased in order to repair the damage; when there is no damage or in healthier conditions, they may remain at baseline or lower expression levels.

As the biggest immune organ, the intestine can secrete some bioactive substances to defend against foreign antigens, toxins, and macromolecules. Paneth cells in the intestinal villi are secretory cells that are specialized in the production of antimicrobial peptides (Rescigno, 2011). In this study, *L. rhamnosus* increased the mRNA expression of antimicrobial peptides *pBD-1* and *PMAP-37*. On the other hand, *LYZ* expression decreased dramatically. LYZ hydrolyzes *N*-acetylmuramic acid and *N*-acetylglucosamine, which are constituents of the peptidoglycan layer of bacterial cell walls (Balcázar et al., 2007). Gram-negative bacteria, such as *Escherichia coli*, are more resistant to LYZ than Gram-positive bacteria, such as *Lactobacillus*, because their outer membranes hinder the access of lysozyme to peptidoglycan (Callewaert and Michiels, 2010). Therefore, we conjecture that due to the elevated antibacterial ability caused by *pBD-1* and *PMAP-37*, there is no need for intestinal mucosa to express more *LYZ* to exert its bactericidal capacity and protect intestinal symbiotic bacteria in suckling piglets.

Innate immunological responses have evolved specifically to defend the intestinal mucosal interface by limiting direct bacterial contact with the epithelial surface. Enterocytes are known to serve as immunoeffector cells and are capable of secreting cytokines and chemokines to regulate inflammation (Kagnoff and Eckmann, 1997). Different strains of *Lactobacillus* species can elicit a wide range of cytokine responses in immune cells (Meijerink et al., 2010, 2012). In our findings, except for IFN-γ, levels of all the tested pro-inflammatory cytokines and chemokine were decreased, whereas levels of IL-10 and TGF-β1, the well-known anti-inflammatory cytokines, were increased in the *L. rhamnosus* group. The result of IFN-γ secretion was in line with previous studies in which *Lactobacillus* augmented the number of IFN-γ producing cells and increased the synthesis of IFN-γ in the small intestine of mice (Wen et al., 2014, 2015), as IFN-γ plays a key role in the maturation of immune cells and regulates their cellular proliferation in the intestine (Rumbo et al., 2004). As for qRT-PCR results, the *IFN-γ* and *TGF-β* transcript levels were in accordance with the ELISA results. Although mRNA expression is predictive for protein expression, in the present study, the *TNFα* and *IL-8* transcription levels were not consistent with the protein secretions. However, in previous studies, the same inconsistency also existed (Brundel et al., 2001; Guo et al., 2008), implying that there may be some post-transcriptional regulation (Brundel et al., 2001). In addition, *MCP-1* transcript level was markedly elevated by *L. rhamnosus*. Similar to our results, probiotic *L. rhamnosus*

GG and *E. coli* Nissle 1917 have also been reported to enhance MCP-1 expression in other studies (Lan et al., 2005; Ukena et al., 2005). MCP-1 is a proinflammatory chemokine produced by many cells, including epithelial, endothelial, and smooth muscle cells (Cushing et al., 1990; Standiford et al., 1991). These cells are important for antiviral immune responses in the peripheral circulation and in tissue (Deshmane et al., 2009). Moreover, MCP-1 shows chemotactic activity for monocytes, basophils, natural killer cells, and T lymphocytes (Ukena et al., 2005). Taken together, in this study, *L. rhamnosus* could regulate immune responses and increase the antibacterial ability in the gut of piglets through altering the production of cytokines and chemokines.

Activation of TLRs provides information about the bacterial census in the intestine and activates expression of secreted antimicrobial proteins to maintain mucosal surface-associated bacterial populations at homeostatic levels (Duerkop et al., 2009). Several studies have showed that strain-specific *Lactobacillus* up-regulated *TLR2* and *TLR9* transcript levels in vitro (Cammarota et al., 2009; Vizoso Pinto et al., 2009). Dogi et al. (2010) also observed that *Lactobacillus* increased the numbers of IFN-γ and TNF-α positive cells via TLR9 in mice Peyer's patches. In this research, we obtained similar results in that *L. rhamnosus* significantly elevated the expression of *MD-2*, *TLR-2*, and *TLR-9*. Noticeably, TRAF6, which is critical for TLR-mediated activation of dendritic cells (Han et al., 2015), was also induced by *L. rhamnosus*. Thus, we conclude that *L. rhamnosus* can regulate the jejunal mucosal immunologic barrier through TLRs in suckling piglets.

5 Conclusions

In conclusion, under normal conditions, oral administration of *L. rhamnosus* GG to newborn piglets is beneficial for the intestinal health of preweaning piglets by improving the biological, physical, and immunologic barriers of intestinal mucosa.

Contributors

Yang WANG and Li GONG performed the experiments and wrote the manuscript. Yan-ping WU and Zhi-wen CUI analyzed the data. Yong-qiang WANG and Yi HUANG edited the manuscript. Xiao-ping ZHANG and Wei-fen LI designed the study.

Compliance with ethics guidelines

Yang WANG, Li GONG, Yan-ping WU, Zhi-wen CUI, Yong-qiang WANG, Yi HUANG, Xiao-ping ZHANG, and Wei-fen LI declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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List of electronic supplementary materials

Table S1 Composition of sows' diet
Table S2 Nutrient levels of sows' diet
Table S3 Composition of pre-starter feed
Table S4 Nutrient levels of pre-starter feed
Fig. S1 Experimental protocol design

中文概要

题　目：口服鼠李糖乳杆菌 GG 影响哺乳仔猪肠道屏障功能的研究

目　的：探究新生仔猪口服鼠李糖乳杆菌 GG 对肠道屏障功能的影响。

创新点：新生仔猪早期口服鼠李糖乳杆菌 GG 可明显改善其断奶前肠道菌群结构及免疫屏障功能。

方　法：二十四头新生仔猪分为对照组和实验组：对照组仔猪在出生后第 1、3、5 天口服 2 mL 0.1 g/mL 的脱脂牛奶；而实验组仔猪口服等体积的含有活鼠李糖乳杆菌 GG 的脱脂牛奶。饲喂 25 天后，收集仔猪血清、肠道粘膜和盲肠内容物等样品。通过分析肠道菌群、紧密连接蛋白和细胞因子等指标，评价鼠李糖乳杆菌对肠道屏障功能的影响。

结　论：在正常生理条件下，新生仔猪口服鼠李糖乳杆菌 GG 可明显改变肠道菌群结构。此外，鼠李糖乳杆菌 GG 还可增加仔猪肠道的通透性，并通过调控抗菌肽、细胞因子和趋化因子的分泌以改善肠道的免疫屏障功能。

关键词：鼠李糖乳杆菌；肠道菌群；肠道物理屏障；肠道免疫屏障；仔猪