

# Effects of *Lactobacillus reuteri* LR1 on the growth performance, intestinal morphology, and intestinal barrier function in weaned pigs

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**ABSTRACT:** The objective of this study was to investigate the effects of *Lactobacillus reuteri* LR1, a new strain isolated from the feces of weaned pigs, on the growth performance, intestinal morphology, immune responses, and intestinal barrier function in weaned pigs. A total of 144 weaned pigs (Duroc × Landrace × Yorkshire, 21 d of age) with an initial BW of  $6.49 \pm 0.02$  kg were randomly assigned to 3 dietary treatments with 8 replicate pens, each of per treatment and 6 pigs. Pigs were fed a basal diet (CON, controls), the basal diet supplemented with 100 mg/kg olaquinox and 75 mg/kg aureomycin (OA) or the basal diet supplemented with  $5 \times 10^{10}$  cfu/kg *L. reuteri* LR1 for a 14-d period. At the end of study, the ADG, ADFI, and G:F were calculated, and 1 randomly selected pig from each pen was euthanized for sample collection. The LR1 increased ADG (22.73%,  $P < 0.05$ ) compared with CON. The villus height of the ileum was increased ( $P < 0.05$ ) and crypt depth in duodenum

was reduced ( $P < 0.05$ ), along with increased ( $P < 0.05$ ) villus height to crypt depth ratio of the jejunum and ileum by LR1 compared with CON and OA. LR1 increased ( $P < 0.05$ ) ileal mucosal content of IL-22 and transforming growth factor- $\beta$  compared with OA. Compared with CON, LR1 increased ( $P < 0.05$ ) and OA decreased ( $P < 0.05$ ) the ileal content of secretory immunoglobulin A (sIgA), and the abundance of transcripts of porcine  $\beta$ -defensin 2 and protegrin 1-5. Compared with CON, LR1 increased ( $P < 0.05$ ) tight junction protein zonula occludens-1 and occludin transcripts in the mucosa of the jejunum and ileum, and those of mucin-2 in ileal mucosa. The relative expression of toll-like receptor 2 (TLR2) and TLR4 were increased ( $P < 0.05$ ) in ileal mucosa in pigs fed LR1 compared with CON. In conclusion, these data indicated that dietary LR1 supplementation at  $5 \times 10^{10}$  cfu/kg improved growth performance, intestinal morphology, and intestinal barrier function in weaned pigs.

**Key words:** antibiotics, intestinal barrier function, *Lactobacillus reuteri* LR1, weaned pig

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## INTRODUCTION

Weaning stress causes intestinal inflammation and intestinal barrier disruptions in pigs, leading to diarrhea and decreased growth performance (Smith et al., 2010; Hu et al., 2013). Although antibiotics have long been used to prevent diarrhea or to promote growth in pigs, increasing bacterial resistance and public health concerns is limiting their use (Ashiru-Oredope and Hopkins, 2015). Additionally, recent studies have showed that antibiotics disrupt the intestinal barrier function by decreasing the expression of tight junction (TJ) proteins, antimicrobial peptides, and mucins in the intestine (Wlodarska et al., 2011b; Yi et al., 2016). However, antibiotics reduction increases postweaning diarrhea, mortality, and feed costs in swine production. There is an urgent need to find alternatives to antibiotics. *Lactobacilli reuteri* strains are important members of the intestinal commensal microbiota (Wegmann et al., 2015). Previous studies in mice showed that *L. reuteri* enhanced the intestinal barrier function by improving expression of TJ proteins (Ahl et al., 2016). In addition, *L. reuteri* I5007 enhanced intestinal barrier function in newborn pigs via improving gene expression of TJ proteins (Hou et al., 2014; Yang et al., 2015). Previous studies in this laboratory have shown that a new strain of *L. reuteri* isolated from the feces of healthy pigs (LR1) showed notable acid and bile resistance, antibacterial activity, and adherence to intestinal porcine epithelial cells, and increased the expression of zonula occludens 1 (ZO-1) in intestinal epithelial cells (Wang et al., 2016). However, the possible effects of *L. reuteri* LR1 on growth performance and intestinal barrier function in weaned pigs in vivo remain unknown. Thus, the objective of this study was to investigate the effects of *L. reuteri* LR1 on the growth performance, intestinal morphology, immune responses, and intestinal barrier function in weaned pigs.

## MATERIALS AND METHODS

All animal protocols used in this study were in accordance with the Guidelines for the Care and Use of Animals for Research and Teaching and approved by the Animal Care and Use Committee of Guangdong Academy of Agricultural Sciences.

### *Animals and Experimental Design*

A total of 144 weaned pigs (Duroc × Landrace × Yorkshire, 21 d of age) with an initial BW of  $6.49 \pm 0.02$  kg were balanced for sex and then

randomly assigned to 3 dietary treatments with 8 replicate pens per treatment, each pen containing 3 barrows and 3 gilts with a complete randomized experimental design. Control pigs (CON) were fed a basal diet, and the others were fed the basal diet supplemented with 100 mg/kg olaquinox plus 75 mg/kg aureomycin (OA), or the basal diet supplemented with  $5 \times 10^{10}$  cfu/kg *L. reuteri* LR1 for a 14-d period. The basal diet was formulated to meet nutritional requirements based on pig BW (NRC, 2012), and the ingredients and composition of the basal diet are listed in Table 1. All pigs were allowed ad libitum access to feed and water throughout. At the end of study, the ADG, ADFI, and G:F were calculated. One randomly selected pig from each pen within a treatment was sacrificed after anesthesia by i.m. injection with sodium pentobarbital (40 mg/kg BW). Samples of the middle duodenum, the middle jejunum, and distal ileum were carefully collected, rinsed with PBS, and fixed in 4% paraformaldehyde. Other segments of the middle jejunum and distal ileum were opened and thoroughly rinsed with sterile normal saline, and then mucosa was collected by scraping with glass slides and immediately snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### *Analysis of Intestinal Morphology*

The fixed intestinal segments were dehydrated, embedded in paraffin, and sectioned for intestinal morphology as described before (Gao et al., 2013). Briefly, sections of 5- $\mu\text{m}$  thickness were deparaffinized in xylene, rehydrated, and stained with hematoxylin and eosin (H&E). Images were obtained using an Axio Scope A1 microscope (Zeiss, Germany). The villous height and crypt depth of each segment were measured with Image-Pro software (Media Cybernetics, Rockville, MD). A minimum of 9 villi from each sample were measured for each treatment. Mean villus height, crypt depth, and villus height to crypt depth ratio for each pig were used for analysis.

### *Intestinal Cytokines Determined by ELISA*

After grinding in liquid nitrogen, total protein was extracted from the jejunal and ileal mucosa samples with a lysis buffer (KeyGEN, Nanjing, China), followed by clarification by centrifugation; protein content in supernatants was determined using a BCA protein assay kit (KeyGEN). The concentrations of IL-6, IL-8, IL-10, and IL-22 were determined in duplicate using porcine ELISA kits (Raybiotech, Norcross, GA) as described before (Yi

**Table 1.** The formulations and chemical composition of the basal diet (as-fed basis)

Ingredient	%
Corn, 8%	35.31
Extrude corn	15.00
Fermented soybean meal, 54%	9.00
Expanded soybean	10.00
Fish meal	4.00
Whey powder	11.00
Soybean hull	5.00
Soybean oil	1.20
Blood plasma	3.00
Sucrose	2.00
Choline chloride	0.20
Salt	0.45
Monocalcium phosphate	0.62
Limestone	0.65
L-Lys HCl	0.60
DL-Met	0.22
L-Thr	0.21
L-Trp	0.04
Premix <sup>1</sup>	1.50
Total	100
Energy and nutrient composition	
DE, kcal/kg	3550
CP (analyzed), %	19.30
Ca (analyzed), %	0.70
Total P (analyzed), %	0.56
STTD of P, %	0.39
Lys (analyzed), %	1.55
Met + Cys, %	0.89
Thr, %	0.97
Trp, %	0.26

<sup>1</sup>Provided vitamin and mineral premix per kg of diet: vitamin A = 2400 IU; vitamin D3 = 2800 IU; vitamin E = 200 IU; vitamin K3 = 5 mg; vitamin B12 = 40 µg; vitamin B1 = 3 mg; vitamin B2 = 10 mg; niacin = 40 mg; pantothenic acid = 15 mg; folic acid = 1 mg; vitamin B6 = 8 mg; biotin = 0.08 mg; Fe (FeSO<sub>4</sub>·H<sub>2</sub>O) = 120 mg; Cu (CuSO<sub>4</sub>·5H<sub>2</sub>O) = 16 mg; Mn (MnSO<sub>4</sub>·H<sub>2</sub>O) = 70 mg; Zn (ZnSO<sub>4</sub>·H<sub>2</sub>O) = 120 mg; I (CaI<sub>2</sub>O<sub>6</sub>) = 0.7 mg; Se (Na<sub>2</sub>SeO<sub>3</sub>) = 0.48 mg.

et al., 2016). In addition, the level of transforming growth factor-β (TGF-β) and secretory IgA (sIgA) was detected in duplicate using porcine ELISA kits (Cusabio Biotech Co., Ltd, Wuhan, China) as described previously (Gao et al., 2013). These data were expressed as pg/mg protein.

### Real-Time PCR for Relative Measurement of Transcripts

Total RNA was extracted from mucosal tissue powders using TRIzol reagent (Invitrogen, Carlsbad, CA). The purity and the yield of the RNA were evaluated using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). The integrity of RNA was determined by electrophoresis in 1% agarose gels, and 1 µg of RNA

was used to generate cDNA in a volume of 20 µL with a PrimeScript RT Reagent Kit (Takara, Dalian, China). Real-time PCR was performed in duplicate with SYBR Green master mix in a CFX Connect Detection system (Bio-Rad, Hercules, CA). Each 10-µL reaction included 5-µL iTaq Universal SYBR Green Supermix (Bio-Rad), 0.5-µL forward primer (10 µM), 0.5-µL reverse primer (10 µM), and 4-µL 20-fold diluted cDNA. The thermocycler protocol consisted of 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C. GAPDH and β-actin were used as house-keeping genes, and the primers for the real-time PCR in this study are listed in Table 2. The relative mRNA expression of the target genes was determined using the 2<sup>-ΔΔCt</sup> method, and data for each target transcript were normalized to the control pigs (1.0).

### Immunofluorescence

Paraformaldehyde-fixed and paraffin-embedded sections of the ileum were used for immunofluorescence examination, as described (Yi et al., 2017). Briefly, sections of 5-µm thickness were deparaffinized and rehydrated and then processed for antigen retrieval. The sections were incubated in the dark for 20 min with 3% hydrogen peroxide. The sections were then incubated sequentially with each primary antibody (rabbit polyclonals, 1:200, anti-ZO-1, Abcam Inc., Cambridge, MA and anti-mucin-2, GeneTex Inc., Irvine, CA) and then with 1:50 CY3-labeled goat antirabbit IgG (Abcam). Nuclei were stained with DAPI. Images were obtained using an ECLIPSE TI-SR microscope with a DS-U3 Image-Pro system (Nikon, Tokyo, Japan).

### Statistical Analyses

Replicate ( $n = 8$ ) served as the experimental unit. Data were analyzed using the general linear model (GLM) with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The effect of dietary treatment was assessed by one-way ANOVA followed by Tukey's test. All data are expressed as the means ± SEM. Differences were considered to be significant at  $P < 0.05$ .

## RESULTS

### Effects of LR1 on the Growth Performance in Weaned Pigs

The LR1 treatment increased ADG (22.7%,  $P < 0.05$ ) above CON (Table 3), similar to the effect of OA on ADFI (17.8%,  $P < 0.05$ ) and ADG (29.6%,  $P < 0.05$ ) compared with CON.

**Table 2.** Primers for real-time PCR in this study

Gene <sup>1</sup>	Sequence (5'–3')	Size(bp)	Accession number
ZO-1	Forward: AGCCCGAGGCGTGT Reverse: GGTGGGAGGATGCTGTTG	147	XM_013993251
Occludin	Forward: GCACCCAGCAACGACAT Reverse: CATAGACAGAATCCGAATCAC	144	XM_005672525
Mucin-2	Forward: CTGCTCCGGGTCCTGTGGGA Reverse: CCCGCTGGCTGGTGCGATAC	100	XM_007465997.1
pBD2	Forward: CCAGAGGTCCGACCACTACA Reverse: GGTCCCTTCAATCCTGTTGAA	88	AY506573.1
PGI-5	Forward: GTAGGTTCTGCGTCTGTGTCG Reverse: CAAATCCTTCACCGTCTACCA	166	XM_005669497.2
NOD1	Forward: ACCGATCCAGTGAGCAGATA Reverse: AAGTCCACCAGCTCCATGAT	140	NM_001114277.1
NOD2	Forward: CCTTTGAAGATGCTGCCTG Reverse: GATTCTCTGCCCATCGTAG	100	NM_001105295.1
TLR2	Forward: TCACTTGCTAACTTATCATCCTCTTG Reverse: TCAGCGAAGGTGTCATTATTGC	162	NM_213761.1
TLR4	Forward: GCCATCGCTGCTAACATCATC Reverse: CTCATACTCAAAGATACACCATCGG	108	NM_001113039.1
β-actin	Forward: CTGCGGCATCCACGAAACT Reverse: AGGGCCGTGATCTCCTTCTG	380	XM_003124280
GAPDH	Forward: ACTCACTCTTCCACTTTTGATGCT Reverse: TGTTGCTGTAGCCAAATTCA	100	NM_001206359

<sup>1</sup>ZO-1 = zonula occludens-1; pBD2 = porcine β defensin 2; PGI-5 = protegrin 1-5; NOD = nucleotide binding oligomerization domain; TLR = toll-like receptor; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

**Table 3.** Effects of LR1 on the growth performance in weaned pigs

Item	CON	OA	LR1	SEM	P value
Initial BW, kg	6.50	6.48	6.49	0.01	0.611
Final BW, kg	9.27 <sup>b</sup>	10.07 <sup>a</sup>	9.89 <sup>a</sup>	0.11	0.002
ADFI, g/d	310 <sup>b</sup>	365 <sup>a</sup>	358 <sup>ab</sup>	11	0.002
ADG, g/d	198 <sup>b</sup>	256 <sup>a</sup>	243 <sup>a</sup>	8	0.065
G:F	0.64	0.70	0.68	0.01	0.148

CON = a basal diet; OA = a basal diet supplemented with 100 mg/kg olaquinox + 75 mg/kg aureomycin; LR1 = a basal diet supplemented with  $5 \times 10^{10}$  cfu/kg *L. reuteri* LR1.

<sup>a,b</sup>Means lacking common superscript letter indicated significant differences ( $P < 0.05$ ) within a row.

### Effects of LR1 on Intestinal Morphology of Weaned Pigs

The LR1 reduced ( $P < 0.05$ ) jejunal crypt depth and both LR1 and OA increased ( $P < 0.05$ ) villus height to crypt depth ratio compared with CON (Table 4). In addition, LR1 increased ( $P < 0.05$ ) both the villus height and the villus height to crypt depth ratio of the ileum over those in CON and OA pigs.

### Effects of LR1 on Intestinal Cytokines of Weaned Pigs

To evaluate the LR1's effects on immune responses in the intestine of weaned pigs, the levels of cytokine IL-6, IL-8, IL-10, IL-22, and TGF-β in the mucosa of jejunum and ileum were determined (Table 5). The LR1 increased ( $P < 0.05$ ) ileal

contents of TGF-β, compared with CON and OA. Additionally, LR1 increased ( $P < 0.05$ ) ileal contents of IL-22 compared with OA.

### Effects of LR1 on Intestinal Barrier Function in Weaned Pigs

Given the roles of IL-22 and TGF-β in intestinal barrier function, the effects of LR1 on sIgA and the expression of genes encoding TJ proteins, antimicrobial peptides, and mucins in the mucosa of the jejunum and ileum were examined. For TJ-related components, LR1 increased ( $P < 0.05$ ) ZO-1 and occludin transcripts in jejunal and ileal mucosa compared with CON (Figure 1A) and OA increased jejunal mucosal expression of ZO-1. When the 2 supplemented diets were compared, LR1 had more ( $P < 0.05$ ) ZO-1 and occludin transcripts in ileal mucosa and occludin transcripts in

**Table 4.** Effects of LR1 on intestinal morphology in weaned pigs

Item	CON	OA	LR1	SEM	<i>P</i> value
<b>Duodenum</b>					
Villus height, $\mu\text{m}$	378	378	413	13	0.447
Crypt depth, $\mu\text{m}$	229	204	217	10	0.392
Villus height/crypt depth	1.68	1.86	1.94	0.07	0.354
<b>Jejunum</b>					
Villus height, $\mu\text{m}$	377	412	421	14	0.403
Crypt depth, $\mu\text{m}$	239 <sup>a</sup>	199 <sup>ab</sup>	167 <sup>b</sup>	11	0.012
Villus height/crypt depth	1.62 <sup>c</sup>	2.13 <sup>b</sup>	2.54 <sup>a</sup>	0.11	0.000
<b>Ileum</b>					
Villus height, $\mu\text{m}$	316 <sup>b</sup>	340 <sup>b</sup>	405 <sup>a</sup>	13	0.004
Crypt depth, $\mu\text{m}$	166	186	154	6	0.140
Villus height/crypt depth	1.95 <sup>b</sup>	1.85 <sup>b</sup>	2.71 <sup>a</sup>	0.14	0.011

CON = a basal diet; OA = a basal diet supplemented with 100 mg/kg olaquinox + 75 mg/kg aureomycin; LR1 = a basal diet supplemented with  $5 \times 10^{10}$  cfu/kg *L. reuteri* LR1.

<sup>a,b,c</sup>Means lacking common superscript letter indicated significant differences ( $P < 0.05$ ) within a row.

**Table 5.** Effects of LR1 on cytokines production in intestines in weaned pigs

Item <sup>1</sup>	CON	OA	LR1	SEM	<i>P</i> value
<b>Jejunal mucosa</b>					
IL-6	5.49	5.36	3.67	0.68	0.499
IL-8	72.80	61.43	42.71	6.57	0.171
IL-10	6.62	7.02	5.36	0.59	0.514
IL-22	4.96	4.17	2.99	0.43	0.170
TGF- $\beta$	18.10	13.84	11.01	1.41	0.115
<b>Ileal mucosa</b>					
IL-6	4.59	4.49	4.99	0.46	0.905
IL-8	51.62	55.41	65.97	4.80	0.472
IL-10	6.76	6.82	9.37	0.77	0.300
IL-22	4.30 <sup>a,b</sup>	3.95 <sup>b</sup>	6.53 <sup>a</sup>	0.49	0.058
TGF- $\beta$	15.51 <sup>b</sup>	15.91 <sup>b</sup>	37.61 <sup>a</sup>	3.87	0.019

CON = a basal diet; OA = a basal diet supplemented with 100 mg/kg olaquinox + 75 mg/kg aureomycin; LR1 = a basal diet supplemented with  $5 \times 10^{10}$  cfu/kg *L. reuteri* LR1. Data are normalized as pg/1 mg protein of tissues.

<sup>1</sup>TGF- $\beta$  = transforming growth factor- $\beta$ .

<sup>a,b</sup>Means lacking common superscript letter indicated significant differences ( $P < 0.05$ ) within a row.

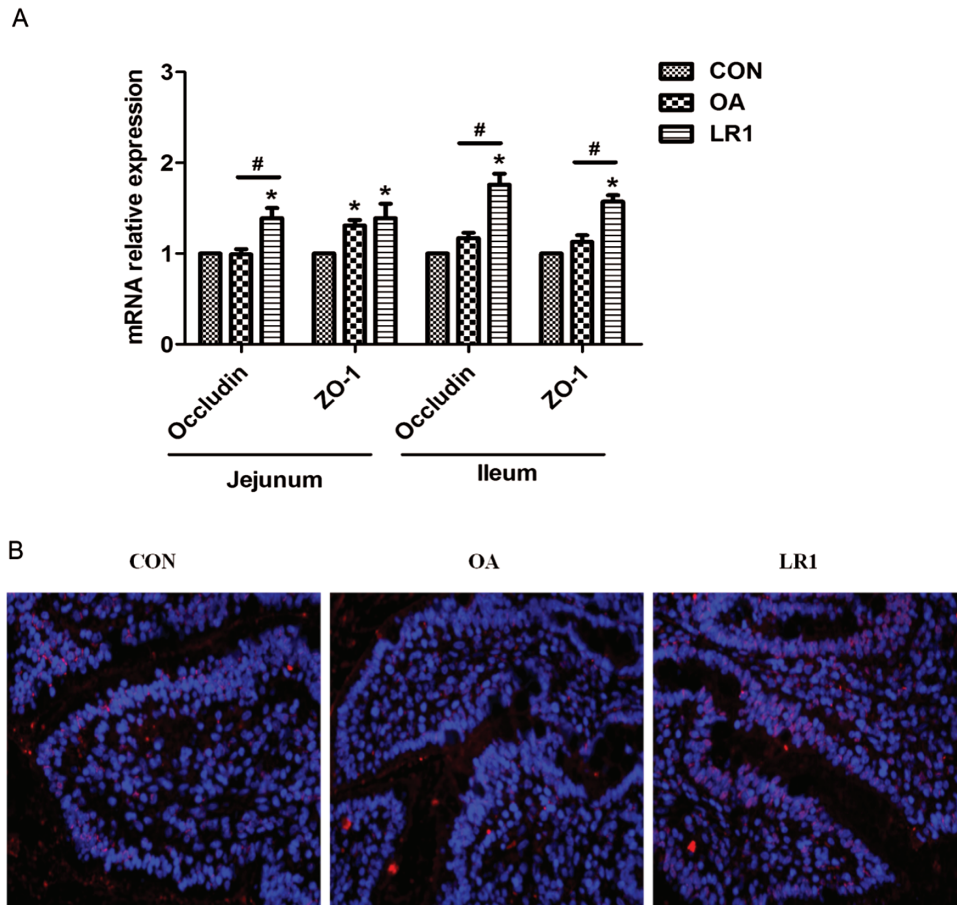
jejunal mucosa. The protein expression of ZO-1 in the ileum was confirmed by immunofluorescence (Figure 1B).

With regard to antimicrobial protein, OA, but not LR1, decreased ( $P < 0.05$ ) the sIgA content of the jejunal mucosa from that in CON (Figure 2A); concentrations of sIgA in jejunal and ileal mucosa in LR1 pigs exceeded ( $P < 0.05$ ) those of OA and were the same or greater than in CON. Transcripts of porcine  $\beta$  defensin 2 (pBD2) and protegrin 1-5 (PG1-5) in jejunal and ileal mucosa were increased ( $P < 0.05$ ) in pigs fed LR1, compared with CON (Figure 2B). However, OA decreased ( $P < 0.05$ ) pBD2 and PG1-5 in ileal mucosa compared with CON. Mucin-2 transcripts in ileal mucosa were greatest ( $P < 0.05$ ) in pigs fed LR1 compared with both CON and OA pigs (Figure 3A). These results on ileal mucin-2 with

LR1 were confirmed at the protein level by immunofluorescence (Figure 3B).

#### **Effects of LR1 on toll-like receptors (TLRs) and nucleotide binding oligomerization domain (NODs) in Intestines of Weaned Pigs**

The possible mechanism through which LR1 influenced intestinal barrier functions was examined using relative transcript abundance of genes encoding toll-like receptor-2 and -4 (TLR2, TLR4), and nucleotide binding oligomerization domains 1 and 2 (NOD1, NOD2) in the intestines of weaned pigs (Figure 4). The expression of NOD2 in jejunal and ileal mucosa was increased ( $P < 0.05$ ) by OA, and both OA and LR1 decreased ( $P < 0.05$ ) NOD1 expression in jejunal mucosa compared with CON (Figure 4A). In terms of TLRs expression, relative



**Figure 1.** LR1 increased the tight junction protein expression in the intestine of weaned pigs. (A) Relative mRNA expression levels of zonula occludens-1 (ZO-1) and occludin in the jejunal and ileal mucosa determined by real-time PCR. (B) The ZO-1 expression in the ileal mucosa confirmed using immunofluorescence. Red, ZO-1; blue, DAPI; 200 $\times$ . All data are expressed as the mean  $\pm$  SEM ( $n = 8$ ). Differences were determined by one-way ANOVA followed by Tukey's test. \* $P < 0.05$  compared with CON, # $P < 0.05$  LR1 compared with OA. CON, a basal diet; OA, a basal diet supplemented with 100 mg/kg olaquinox + 75 mg/kg aureomycin; LR1, a basal diet supplemented with  $5 \times 10^{10}$  cfu/kg *L. reuteri* LR1.

abundance of TLR2 and TLR4 transcripts in jejunal mucosa was increased ( $P < 0.05$ ) in pigs fed LR1 compared with CON (Figure 4B). Comparing the supplements, TLR2 in the jejunal mucosa was greater ( $P < 0.05$ ) in those given LR1 than OA, whereas OA decreased ( $P < 0.05$ ) TLR2 transcripts in ileal mucosa. Compared with CON, TLR2 expression in ileal mucosa increased ( $P < 0.05$ ) in LR1 pigs but that of TLR4 decreased ( $P < 0.05$ ).

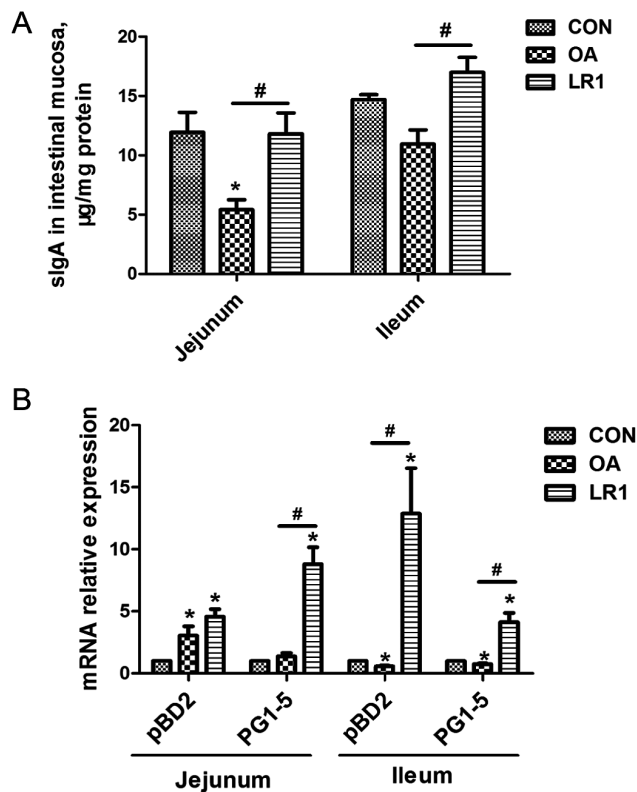
## DISCUSSION

In this study, dietary LR1 supplementation at  $5 \times 10^{10}$  cfu/kg could improve the growth performance, intestinal morphology, and intestinal barrier function in weaned pigs. Antibiotics increased the growth performance of weaned pigs, but reduced the sIgA production and antimicrobial peptides expression in the intestine.

Aureomycin and olaquinox have been used to improve the growth performance of pigs during weaning in the past years in China. In this study,

dietary supplementation with 75 mg/kg aureomycin plus 100 mg/kg olaquinox increased the ADG and ADFI in weaned pigs. However, the use of the antimicrobial growth promoter olaquinox has been banned in the European Union since September 1999 and in China since August 2017 because of its side effects on animals. To overcome the increased rate of mortality, morbidity, and post-weaning diarrhea because of this ban, a number of alternatives have been proposed. *Lactobacillus reuteri* LR1 is a new strain of *L. reuteri* isolated from the feces of healthy weaned pig in our previous study (Wang et al., 2016). Surprisingly, the LR1's improvements of ADG of weaned pigs are similar to the antibiotics in this study. These data indicate that the LR1 may be an effective alternative to antibiotics for the improvement of growth performance in weaned pigs.

*Lactobacillus reuteri* is an important member of the commensal microbiota in intestines of both humans and animals. *Lactobacillus reuteri* has exhibited to regulate immune response and improve



**Figure 2.** LR1 improved the antimicrobial proteins expression in the intestine of weaned pigs. (A) Levels of sIgA in the jejunal and ileal mucosa determined by ELISA. (B) Relative mRNA expression levels of pBD2 and PG1-5 in the jejunal and ileal mucosa determined by real-time PCR. All data are expressed as the mean  $\pm$  SEM ( $n = 8$ ). Differences were determined by one-way ANOVA followed by Tukey's test. \* $P < 0.05$  compared with CON, # $P < 0.05$  LR1 compared with OA. CON, a basal diet; OA, a basal diet supplemented with 100 mg/kg olaquinox + 75 mg/kg aureomycin; LR1, a basal diet supplemented with  $5 \times 10^{10}$  cfu/kg *L. reuteri* LR1.

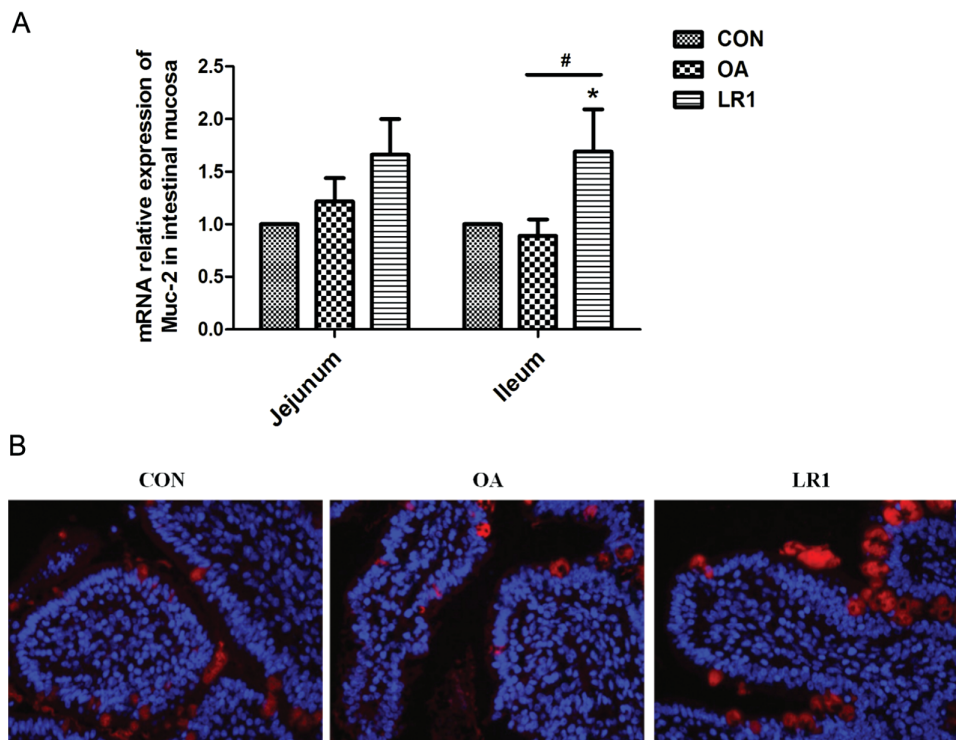
the gut health (Li et al., 2008; Liu et al., 2013). A recent study showed that *L. reuteri* together with a tryptophan-rich diet could reprogram intraepithelial CD4<sup>+</sup> T cells into immunoregulatory T cells (Cervantes-Barragan et al., 2017). In addition, *L. reuteri* could reduce intestinal inflammation via inhibiting TLR4-nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling pathway (Liu et al., 2012). In this study, LR1 increased the production of cytokines IL-22 and TGF- $\beta$  associated with inhibiting TLR4 expression in the ileum of weaned pigs. Previous studies showed that TGF- $\beta$  down-regulates inflammatory cytokine production in monocytes and macrophages (Laine et al., 2004) and plays a role in the induction from CD4<sup>+</sup> T cells of induced immune regulatory T cells (Stifter et al., 2016). IL-22 is produced by activated NK and T cells, and initiates innate immune responses against pathogens invasion especially in the intestinal epithelial cells (Trevejo-Nunez et al., 2016). Importantly, TGF- $\beta$  and IL-22 also play important roles in maintaining the integrity of intestinal barrier and wound healing in the intestine

(Howe et al., 2005; Hainzl et al., 2015; Xiao et al., 2017). Thus, LR1's effects on the intestinal barrier function were determined in the next.

The intestinal epithelial barrier is the first line of restricting the invasion of microbes, food antigens, and toxins. Weaning stress causes intestinal barrier damage associated with decreasing TJ proteins expression in pigs (Hu et al., 2013; Yi et al., 2016). Tight junction proteins create semipermeable barriers that can separate different substances in the epithelial cells of the intestine (Dokladny et al., 2016). ZO-1 and occludin are the most important components of TJ proteins (Han et al., 2015). In this study, dietary supplementation with LR1 increased the expression of ZO-1 and occludin in the intestine of weaned pigs. A previous study showed that *L. reuteri* LR1 attenuated enterotoxigenic *Escherichia coli* (ETEC) K88-induced decreases of the expression of ZO-1 and occludin in porcine intestinal epithelial cells (Wang et al., 2016). In addition, *L. reuteri* I5007, another strain of porcine *L. reuteri*, increased TJ proteins expression in the intestinal epithelial cells in vitro and in the intestine of newborn pig in vivo (Hou et al., 2014; Hou et al., 2015; Yang et al., 2015). *Lactobacillus reuteri* has also shown to improve intestinal epithelial barrier function by promoting cell migration (Preidis et al., 2012). These data indicated that LR1 could improve TJ protein ZO-1 and occludin expression in the intestine.

The mucins and antimicrobial proteins that are secreted by intestinal epithelial cells provide defenses against pathogen invasion into the underlying lamina propria (Wlodarska et al., 2011a; Gallo and Hooper, 2012). In this study, LR1 increased pBD2, PG1-5, and mucin-2 expression in the intestine of weaned pigs. Consistently, previous studies showed that *L. reuteri* I5007 also increased intestinal antimicrobial peptides in pigs (Yang et al., 2015). By contrast, the antibiotics reduced the expression of sIgA, pBD2, and PG1-5 in the intestine of weaned pigs. A previous study reported that administration of the antibiotics neomycin, vancomycin, and metronidazole decreased the expression levels of antimicrobial proteins in intestinal epithelial cells (Brandl et al., 2008). A recent study demonstrated that antibiotics compromised goblet cell function and inner mucus layer production in the intestine (Wlodarska et al., 2011a). All these data indicate that the intestinal barrier function could be improved by LR1 in weaned pigs.

To explore possible mechanisms of intestinal barrier function improved by LR1, the expression of NODs and TLRs in the intestine of weaned



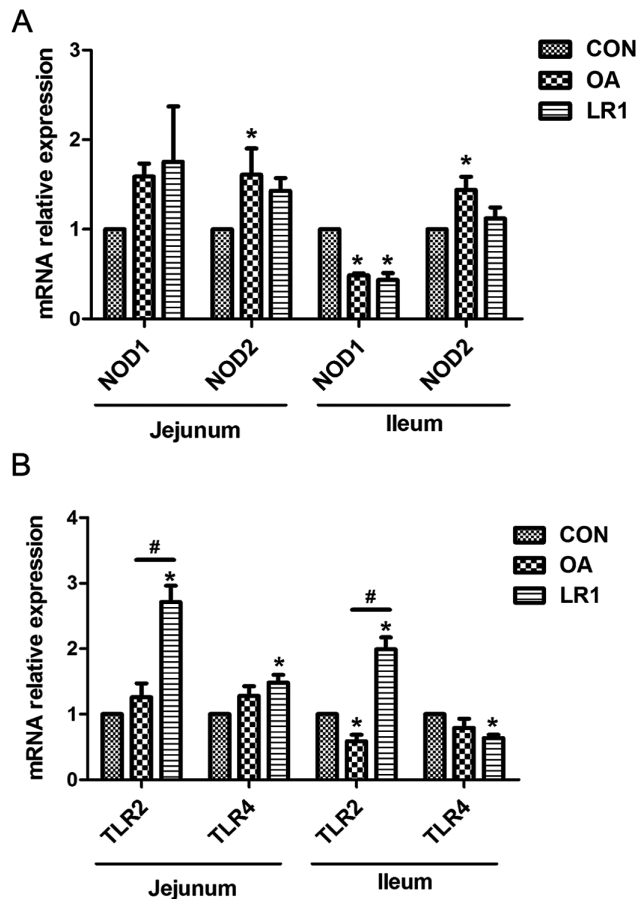
**Figure 3.** LR1 increased mucin-2 expression in the intestine of weaned pigs. (A) Relative mRNA expression level of mucin-2 in the jejunal and ileal mucosa determined by real-time PCR. All data are expressed as the mean  $\pm$  SEM ( $n = 8$ ). Differences were determined by one-way ANOVA followed by Tukey's test. \* $P < 0.05$  compared with CON, # $P < 0.05$  LR1 compared with OA. (B) The mucin-2 expression in the mucosa of the jejunum was confirmed using immunofluorescence. Red, mucin-2; blue, DAPI; 200 $\times$ . CON, a basal diet; OA, a basal diet supplemented with 100 mg/kg olaquinox + 75 mg/kg aureomycin; LR1, a basal diet supplemented with  $5 \times 10^{10}$  cfu/kg *L. reuteri* LR1.

pigs was examined. The NODs and TLRs play important roles in recognizing bacteria and signaling translocation, and then initiating the immune responses in the intestine. The TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, and the TLR2 detects lipoproteins and peptidoglycans in both Gram-positive and Gram-negative bacteria, as well as lipoteichoic acid from Gram-positive bacteria (Kawai and Akira, 2011). In this study, LR1 increased the TLR2 expression but decreased TLR4 expression in the ileum, whereas antibiotics decreased the TLR2 expression. A previous study exhibited that *L. plantarum* increased the mRNA expression of ZO-1 and occludin associated with increasing the TLR2 expression in the intestine of ETEC K88-induced pigs (Yang et al., 2014). Previous studies have showed that the activation of TLR2 could enhance the expression of antimicrobial peptides and TJ proteins (Cario et al., 2004; Lai et al., 2010). Thus, these data suggested that LR1's improvements of antimicrobial peptides and TJ proteins may be via the activation of TLR2. However, the underlying mechanisms are still needed to be addressed in the future.

In this study, both LR1 and antibiotics improved growth performance and intestinal morphology in weaned pigs. Additionally, LR1 increased the

expression of TJ proteins, mucin-2, antimicrobial peptides, and TLR2 in the intestine, whereas antibiotics decreased the expression of sIgA, antimicrobial peptides, and TLR2 expression in the intestine of weaned pigs. These results may come from the differential mode of action between probiotics and antibiotics. Antibiotics are known to modify the composition of the intestinal microbiota including both pathogenic and commensal bacteria, and their improvements of growth performance may come from reduced total intestinal biota biomass and elimination of the harmful bacteria, which compete for nutrients with the host animal (Heo et al., 2013). In this study, the decreased sIgA production and antimicrobial peptide expression may come from reduced pathogenic and total bacterial content in the intestine of pigs fed OA. Although their mode of action has not been fully elucidated, probiotics are known to inhibit pathogen adhesion by competitive exclusion, produce bacteriosins and organic acids against pathogens, and modulate the immune system in the intestine (Gresse et al., 2017). The different regulation of TLR2, sIgA, and antimicrobial peptides may be due to the changes of intestinal microbiota in pigs fed LR1 and antibiotics. Previous studies showed that probiotics improved intestinal barrier function through regulating intestinal microbiome (Preidis et al., 2012).





**Figure 4.** Effects of LR1 on the expression of NODs and TLRs in the intestine of weaned pigs. (A) Relative mRNA expression levels of nucleotide binding oligomerization domain 1 (NOD1) and NOD2 in the mucosa of the jejunum and ileum. (B) Relative mRNA expression levels of toll-like receptor 2 (TLR2) and TLR4 in the mucosa of the jejunum and ileum. All data are expressed as the mean  $\pm$  SEM ( $n = 8$ ). Differences were determined by one-way ANOVA followed by Tukey's test. \* $P < 0.05$  compared with CON, # $P < 0.05$  LR1 compared with OA. CON, a basal diet; OA, a basal diet supplemented with 100 mg/kg olaquinox + 75 mg/kg aureomycin; LR1, a basal diet supplemented with  $5 \times 10^{10}$  cfu/kg *L. reuteri* LR1.

The LR1 could affect intestinal microbiome and then improve intestinal barrier function. However, the limitation of the current study is lacking the microbiome data in the intestine in pigs fed LR1 and antibiotics. The effects and underlying mechanisms of LR1 and antibiotics on intestinal microbiome will be further addressed in our next studies.

In conclusion, these data suggested that dietary LR1 supplementation at  $5 \times 10^{10}$  cfu/kg could improve the growth performance, intestinal morphology, and intestinal barrier function in weaned pigs. The underlying mechanisms of the differential regulation of intestinal barrier function by LR1 and antibiotics are needed to be explored in the future.

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