

## PAPER

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# Oral administration of *Lactobacillus delbrueckii* enhances intestinal immunity through inducing dendritic cell activation in suckling piglets†

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*Lactobacillus delbrueckii* (LAB) has been demonstrated to exert versatile beneficial effects on modulating intestinal immunity, increasing gut microbial diversity, promoting growth performance, and even preventing disease onset in pigs. However, the underlying mechanism of LAB-mediated gut immunity regulation in piglets remains unclear. In this study, we found that supplementation of LAB significantly increases serum TNF- $\alpha$ , ileum IL-4, and IL-10 levels compared with the control group. Meanwhile, oral supplementation of LAB-modified gut microbial communities was evidenced by the increased abundance of the *Lactobacillus* genus in the colon. Mechanistically, LAB induced dendritic cell (DC) maturation and activation, which may be relevant to the activation of NF- $\kappa$ B and MAPK signaling pathways. Moreover, we found that oral administration of LAB during the suckling period shows long-lasting immunomodulatory impacts on intestinal immunity after weaning. Collectively, this study uncovers the mechanism of LAB in regulating the intestinal immunity of piglets, suggesting that LAB can be developed as an immunoenhancing biological agent during the suckling period.

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## 1 Introduction

Over the past few decades, the pre-weaning mortality of piglets has remained a concern of the pig industry worldwide. Many risk factors including low birth weight and impaired development of an embryo and fetus have been reported for the survivability of piglets. However, gut dysbiosis caused by infection remains to be a major non-negligible cause for the pre-weaning mortality of piglets.<sup>1</sup> Due to the immature development of the immune system, piglets are unable to mount an appropriate immune response against invaders, which renders piglets more susceptible to infection or other diseases.<sup>2</sup> Although the colostrum or milk intake provides newborn piglets with immunological protection,<sup>3</sup> it remains incapable of endowing newborns with enough immunity against various insults. Thus, how to promote the development of the

immune system and enhance piglet immunity is important and necessary for pig production.

Probiotics have been attracting increasing attention due to their immunomodulatory function with few side effects. Many studies have demonstrated the beneficial effects of *Lactobacillus* on the maintenance of gut homeostasis.<sup>4,5</sup> As a normal resident microflora in the gastrointestinal tract of humans and animals, the colonization and abundance of *Lactobacillus* contribute to the development and function of intestinal immune cells, and thus could further impact the pathogenesis of intestinal diseases such as inflammatory bowel disease (IBD).<sup>6–8</sup> For example, *Lactobacillus* has been reported to regulate the differentiation of CD4<sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> T cells<sup>9</sup> and CD4<sup>+</sup>FoxP3<sup>+</sup> T regulatory cells,<sup>10</sup> increase the numbers and effector functions of distinct B cell subsets in Peyer's patches (PPs),<sup>11</sup> and promote the maturation and function of dendritic cells (DCs) and macrophages.<sup>10,12</sup> Mechanistically, probiotics alleviate intestinal inflammation *via* modulation of toll-like receptor (TLR)-myeloid differentiation factor 88 (MyD88)-dependent mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF- $\kappa$ B) signaling.<sup>13–15</sup> Therefore, using probiotics including *Lactobacillus* to enhance intestinal immunity and health has a promising prospect.

*Lactobacillus delbrueckii* (LAB), which is a *Lactobacillus* species, plays various beneficial probiotic roles in humans and animals. Our previous study has shown that LAB enhances

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anti-oxidant capacities, increases gut microbial community, and decreases diarrhea rates, and consequently improves the growth performance of pigs.<sup>16–20</sup> In addition, we found that LAB could enhance intestinal mucosal cytokine and secretory IgA (sIgA) production in piglets.<sup>16,19</sup> However, the underlying mechanism of LAB-mediated intestinal immunity regulation remains unclear.

Here, we show that oral administration of LAB increases immune-related cytokine production and induces DC maturation and activation in suckling piglets, which is related to the activation of MAPK and NF- $\kappa$ B signaling. Notably, we found that the immunomodulatory properties of LAB during the suckling period exhibit long-term effects on intestinal immunity during the weaned period. Our data revealed the potential mechanism of LAB in regulating intestinal immunity, suggesting that LAB can be developed as an immuno-enhancing biological feed additive.

## 2 Materials and methods

### 2.1 Preparation and culture of LAB

The strain *Lactobacillus delbrueckii* (LAB, CCTCC M 207040) used in this study was preserved in the microbiology laboratory of the College of Animal Science and Technology, Hunan Agricultural University. LAB was cultured at 37 °C for 48 h in DeMan, Rogosa, and Sharp (MRS) medium under anaerobic conditions. The viable counts in the culture medium were determined by the gradient dilution coating method based on our previous study,<sup>16</sup> and then adjusted to  $5 \times 10^9$  colony-forming units (CFU) per mL. An appropriate dose of LAB was applied for oral gavage to suckling piglets. For cell stimulation, LAB was heat-killed at 56 °C for 60 min.

### 2.2 Animals and experimental design

To determine the effect of LAB supplementation on intestinal immunity during the suckling period, eighty neonatal piglets (Duroc  $\times$  landrace  $\times$  large Yorkshire) from 10 litters were randomly assigned to the control group (CON) and LAB group (LAB) based on the litter origin and body weight (BW), and each group has 5 litters with 8 piglets (4 females and 4 males). The piglets from the LAB group were intragastrically administered with LAB at amounts of 1, 2, 3, and 4 mL ( $5 \times 10^9$  CFU per mL per pig) at 1, 3, 7, and 14 days of age, respectively. The piglets from the CON group received the sterilizing saline. At 21 days of age, 6 piglets in each group were sacrificed for sample collection.

To test whether LAB treatment during the suckling period exhibited long-lasting effects on intestinal immunity after weaning, we treated suckling piglets with LAB as above. After the piglets were weaned at 21 days of age, the piglets from the CON and LAB groups were randomly divided into 6 pens (3 pens per group) with 5 pigs in each pen. These weaned piglets were fed with creep feed and managed until 49 days of age. Among the experimental period, 6 piglets were sacrificed at 28 and 49 days of age for sample collection.

All suckling piglets had free access to sow milk and drinking water, and were housed in an environmentally controlled farrowing cage with hard plastic slatted flooring. All weaned piglets were housed in a temperature-controlled nursery and had *ad libitum* access to feed and water. The creep diet was corn-soybean meal-based as reported in our previous study.<sup>16</sup>

All samples were collected and prepared based on our previous study.<sup>18</sup> Briefly, the serum samples were collected by venipuncture into 10 mL tubes and centrifuged at 3000g for 10 min at 4 °C. About 2 g of colonic contents were taken from the middle of the colon from the piglets for analysis of microbial diversity. Segments (8 cm) of the jejunum and ileum were flushed with ice-cold phosphate-buffered saline thoroughly for mucosal sample preparation.

### 2.3 DC2.4 cells culture and stimulation

DC2.4 cells (the dendritic cell line) were cultured in RPMI-1640 medium supplemented with 10% FBS (fetal bovine serum) and antibiotics (100 U mL<sup>-1</sup> penicillin and 100  $\mu$ g mL<sup>-1</sup> streptomycin) at 37 °C in a 5% CO<sub>2</sub> incubator.

In the cell stimulation experiment,  $1 \times 10^6$  DC2.4 cells were plated in 6-well cell culture plates. The cells were stimulated with lipopolysaccharides (LPS), a low dose of heat-killed (LAB-HK-L), live (LAB-L) LAB ( $1 \times 10^6$ , MOI = 1), a high dose of heat-killed (LAB-HK-H), or live (LAB-H) LAB ( $1 \times 10^7$ , MOI = 10). Then, the cells were collected at indicated time points for further analysis.

### 2.4 Enzyme-linked immunosorbent assay (ELISA) for cytokine measurements

After LPS, heat-killed, or live LAB stimulation for 24 hours, the supernatants of the DC2.4 cells were collected for the ELISA assay. The jejunal and ileal mucosa were homogenized with ice-cold normal saline. After centrifugation (3000g for 10 min at 4 °C), the supernatant of each sample was collected for the ELISA assay. The protein levels of TNF- $\alpha$ , IL-12, IL-2, IL-10, IFN- $\gamma$ , CCL20, CX3CL1, and IL-4 were measured by using ELISA kits (ELISA kit's information is listed in ESI Table 1†) according to the manufacturer's protocol.

### 2.5 Flow cytometry

Fluorescence-coupled antibodies for flow cytometry including FITC-MHC-II, PE-CD80, and APC-CD86 were obtained from Abcam Plc. After LPS, heat-killed, or live LAB stimulation, DC2.4 cells were collected and washed. For surface staining, the cells were washed and stained with fluorescence-conjugated antibodies for 30 minutes at 4 °C. After being washed with PBS, the cells were run on a BD LSRFortessa (BD Immunocytometry Systems), and data were analyzed with FlowJo (Tree Star).

### 2.6 Quantitative real-time PCR

Total RNA from DC2.4 cells, jejunum, or ileum tissues was isolated using TRIzol (Invitrogen) and then treated with DNase I



(Invitrogen, USA). A total of 1 µg of RNA was transcribed using PrimeScript™ RT Master Mix (Takara, RR036A). RT-PCR was performed using Power SYBR Green PCR Master Mix (TaKaRa, RR420A). The amounts of the transcript were normalized to those of the house-keeping gene *Gapdh*. The primers used in this study are listed in ESI Table 2.†

## 2.7 Western blot

Approximately 100 mg of each jejunum and ileum sample were homogenized and then lysed in ice-cold radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Beijing, China) supplemented with phosphatase inhibitor and protease inhibitor cocktail tablets (Roche Diagnostics Ltd, Shanghai, China) based on a previous report.<sup>21</sup>

DC2.4 cells were stimulated with LPS, heat-killed, or live LAB, and the cells were collected with RIPA lysis buffer. After centrifugation (12 000g for 10 min at 4 °C), the supernatants were collected for further analysis.

Approximately 50 µg protein of each sample was used for western blot analysis based on a previous study.<sup>22</sup> The primary and secondary antibodies used in this study are listed in ESI Table 3.†

## 2.8 16S rRNA sequencing for analyzing the microbiota composition

The colonic contents were collected after the piglets were sacrificed. Total genome DNA from each colonic sample was extracted according to the previous study.<sup>23</sup> After detection by 1% agarose gel electrophoresis, the extracted genome DNA was sent to Shanghai Personalbio Technology Co., Ltd for 16S rRNA sequencing. After amplification using a specific primer with the barcode (16S V3 + V4), sequencing was performed on the Illumina Miseq platform. A phylogenetic tree and OTU table were obtained from the Mothur Bayesian classifier. Sequencing libraries were generated and sequencing data were processed according to the previous study.<sup>5,24</sup> Principal Coordinate Analysis (PCoA) was performed to get principal coordinates and visualize from the complex, multidimensional data. The indices of Shannon, Simpson, Chao1, and ACE were used to evaluate the complexity of species' alpha diversity. Predicted functional analysis of the colonic microbiota was performed by PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). The OTUs, alpha diversity, and beta diversity analysis, and microbiota structural analysis were done according to our previous study.<sup>25</sup> Statistical comparison of the relative abundance of the colonic microbiota was performed using a two-tailed unpaired Student's *t*-test to identify the differences in bacterial taxa between the CON and LAB groups. The analysis procedures of the colonic microbiota were processed *via* the Majorbio Cloud Platform (Shanghai Personalbio Technology Co., Ltd, China). The raw reads in this study were uploaded to the National Center of Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under accession number PRJNA792735.

## 2.9 Statistical analysis

All values in the paper are given as means ± SEM unless stated otherwise. Significant differences between the two groups were analyzed with a two-tailed unpaired *t*-test. Multiple groups were analyzed by one-way analysis of variance (ANOVA). All statistical analyses were performed using the GraphPad Prism software (GraphPad). Statistical significance was set on the basis of the *P*-value. N.S., *P* > 0.05; \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

# 3 Results

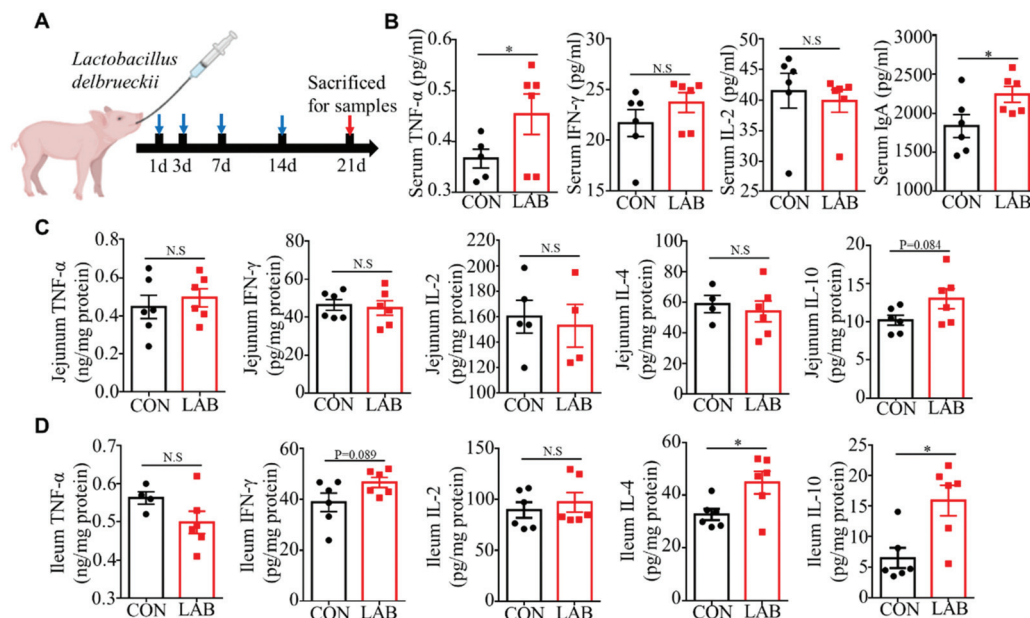
## 3.1 Oral administration of LAB enhances intestinal immunity in suckling piglets

Many studies have shown that probiotics improve intestinal immunity.<sup>6</sup> Consistent with previous reports, we found that the oral supplementation of LAB (Fig. 1A, experimental design) significantly increases the serum TNF-α and IgA levels, while having no impacts on the serum IL-2 and IFN-γ levels compared with the CON group (Fig. 1B). Next, we assessed immune responses in jejunum and ileum from the piglets treated with LAB. We observed that the LAB treatment exhibits no effects on TNF-α, IFN-γ, IL-2, and IL-4 production, except for a tendency to increase the IL-10 levels in the jejunum (Fig. 1C). Moreover, the LAB treatment markedly increased the IL-10 and IL-4 protein levels (Fig. 1D) and had a tendency to increase the IFN-γ production in the ileum (Fig. 1D), but the secretion of IL-2 and TNF-α was unaffected in the jejunum and ileum (Fig. 1D). Collectively, these data suggested that the oral administration of LAB enhances the intestinal immunity in suckling piglets.

## 3.2 Effect of LAB on the gut microbiota in suckling piglets

Microbiota play a critical role in the development of the immune system and induction of the appropriate immune response.<sup>26,27</sup> As newborns are directly accessible to environmental microbiota, the ecosystem of the gut in newborns is easily shaped by various factors.<sup>28</sup> Therefore, we determined whether the LAB treatment was able to shape the gut microbiota in suckling piglets. We collected colonic contents from the piglets in the CON and LAB groups for 16S rRNA sequencing to analyze the colon microbiota composition. An average of 12 265 ± 1953 raw reads was generated from each sample. After removing the low-quality sequences, 12 179 clean tags were clustered into OTUs. A total of 826 OTUs were found in these two groups, but we failed to observe a difference in the number of OTUs between the CON and LAB groups (Fig. S1A†). The Venn diagram of bacterial communities showed that the core OTUs of these groups were 473, and the unique OTUs of the CON and LAB group were 171 and 182, respectively (Fig. S1B†). We then analyzed the alpha-diversity of the microbiota using Shannon, Chao1, and Simpson indexes and found no remarkable differences between the CON and LAB groups (Fig. 2A). Similarly, principal coordinate analysis (PCoA) failed to show distinct clusters in the beta-diversity of the gut microbiota in these two groups (Fig. 2B and Fig. S1C†).





**Fig. 1** Oral administration of LAB enhances intestinal immunity in suckling piglets. (A) Schematic diagram of the experimental design. (B) Serum TNF- $\alpha$ , IgA, IL-2, and IFN- $\gamma$  levels in piglets from the CON and LAB groups. (C and D) Protein levels of TNF- $\alpha$ , IFN- $\gamma$ , IL-2, IL-10, and IL-4 in the jejunum (C) and ileum (D) of piglets. Bar graphs show mean  $\pm$  SEM. N.S.  $P > 0.05$ , \* $P < 0.05$ , \*\* $P < 0.01$ .

The overall microbial composition was further analyzed at the phylum, order, and genus levels. At the phylum level, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the most dominant phyla represented in each group (Fig. 2C), which accounted for more than 96% of the total bacteria in the colon. However, the LAB treatment failed to affect the relative abundance of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* in the colon compared with that in the CON group (Fig. 2D). Similarly, the ratio of *Firmicutes* to *Bacteroidetes* was comparable between these two groups (Fig. 2D). At the order level, oral administration of LAB significantly increased the abundance of *Lactobacillales*, but had no impact on the abundance of other bacterial communities, such as *Clostridiales* and *Bacteroidales* (Fig. 2E and F). Although the majority of the genera were comparable between these two groups at the genus level, the LAB treatment potentially increased the relative abundance of *Lactobacillus* in the colon compared with that in the CON group (Fig. 2G and H). In contrast, oral administration of LAB had a tendency to decrease the abundance of *Bacteroides* compared with that in the CON group (Fig. 2G and H). However, the relative abundance of *Prevotella* was comparable between the CON and LAB groups (Fig. 2G and H).

We further performed PICRUST analysis to evaluate the predicted functions of the microbiota mainly including cellular processes, environmental information processing, genetic information processing, human diseases, metabolism, and organismal systems (Fig. S1D†). KEGG pathways showed that the microbiota-mediated genes were mainly enriched in translation, replication and repair, nucleotide metabolism, amino acid metabolism, energy metabolism, membrane transport, metabolism of cofactors and vitamins, and carbohydrate

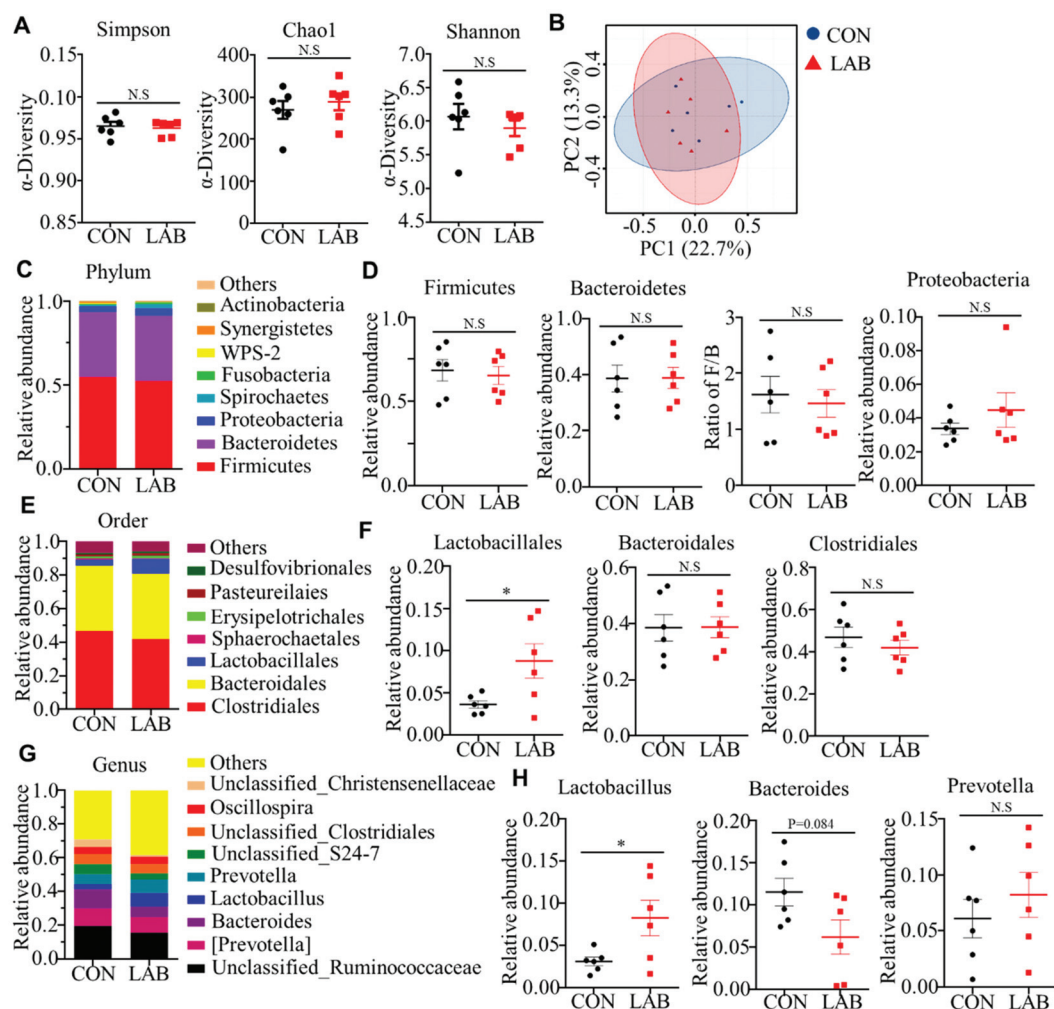
metabolism. However, oral administration of LAB did not influence these functional processes (Fig. S1D†).

### 3.3 Oral administration of LAB induces DC maturation and activation

Intestinal DCs, functioning as professional antigen presenting cells (APCs), constantly sample the luminal content to monitor for pathogens and commensal bacteria. After sampling antigens, the DCs become mature characterized by higher costimulatory molecule expression, and further mediate the innate and adaptive immune response.<sup>29,30</sup> Previous data suggested that oral administration of LAB improves intestinal immunity in suckling piglets.<sup>16</sup> Thus, we hypothesized that LAB may regulate intestinal immunity through inducing DC activation. We first detected the expression of DC maturation markers including CD80, CD86, and MHC-II, and found that oral administration of LAB dramatically upregulated the *CD80*, *CD86*, and *MHC-II* expressions in the jejunum and ileum (Fig. 3A and Fig. S2A†). We also analyzed the expression of chemokine *Ccl20* and its receptor *Ccr6* that are critical to DC recruitment.<sup>31</sup> We observed notably higher *Ccl20* and *Ccr6* expressions in the jejunum and ileum in the LAB group (Fig. 3B and Fig. S2B†). Consistently, the LAB treatment tended to increase the serum CCL20 protein levels, but not the CX3CL1 protein levels (Fig. 3C). These *in vivo* data implied that the LAB treatment may induce DC maturation and activation to regulate intestinal immunity. In support of this, we observed that both heat-killed or live forms of LAB treatment induced higher expressions of CD86, CD80, and MHC-II in DC2.4 cells (Fig. 3D and Fig. S2C†). Interestingly, this effect was more prominent in the live LAB treatment compared to







**Fig. 2** Effect of LAB on gut microbiota in suckling piglets. (A) Shannon, Chao1 and Simpson index in the  $\alpha$ -diversity analysis of microbiota in the colon of piglets from the CON and LAB groups. (B) PCoA plot analysis from each sample in the colon of piglets. (C–H) Microbiota compositions at the phylum level (C and D), order level (E and F), and genus level (G and H) in the colon of piglets. Bar graphs show mean  $\pm$  SEM. N.S.  $P > 0.05$ , \* $P < 0.05$ , \*\* $P < 0.01$ .

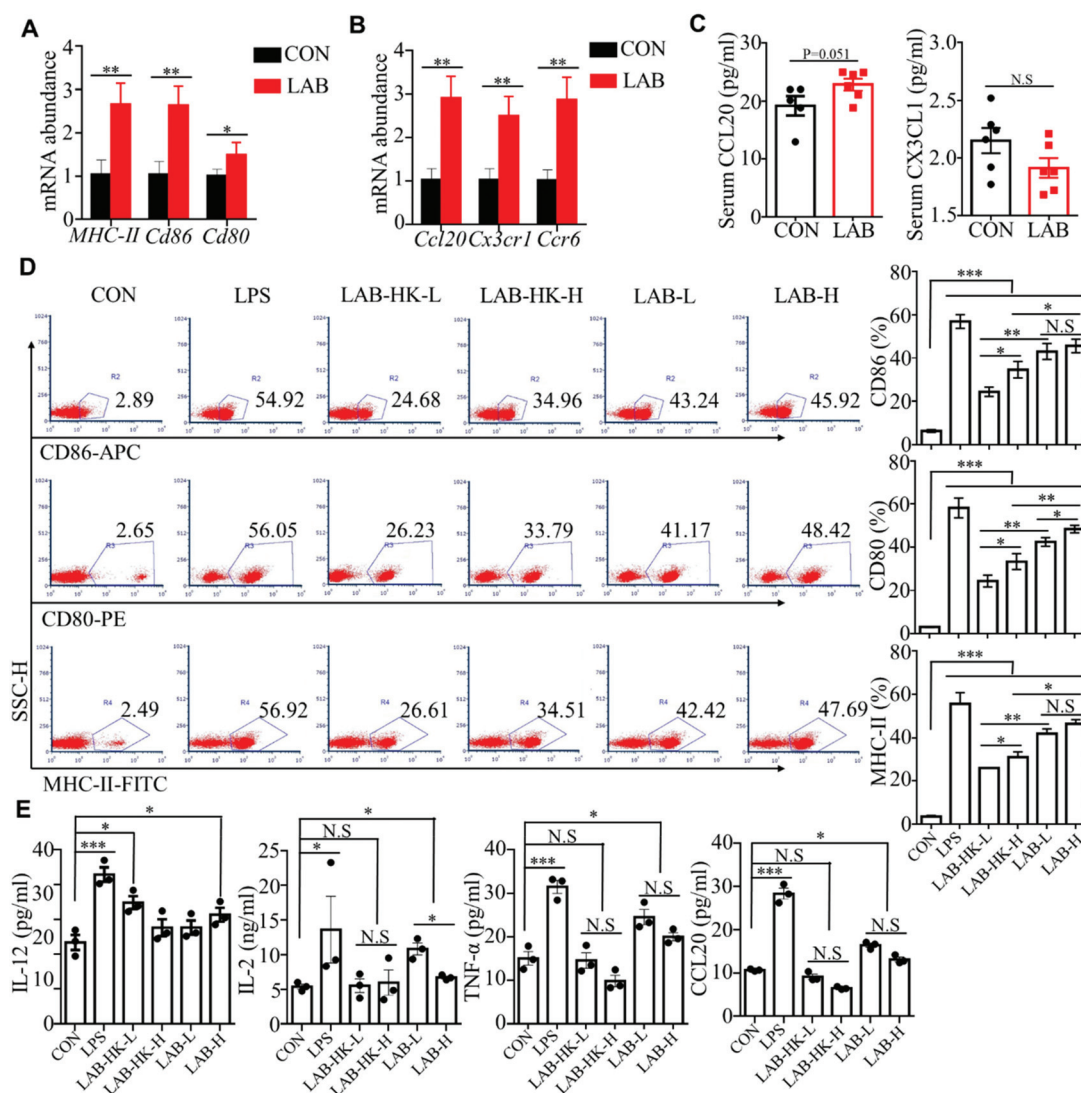
the heat-killed LAB treatment (Fig. 3D). Furthermore, LPS stimulation notably increased IL-12, IL-2, TNF- $\alpha$ , and CCL20 production in DC2.4 cells compared with the control group (Fig. 3E). Meanwhile, the treatment with live, but not heat-killed LAB induced IL-12, IL-2, TNF- $\alpha$ , and CCL20 production in DC2.4 cells (Fig. 3E). Collectively, these data suggested that LAB may regulate intestinal immunity by inducing DC maturation and activation.

### 3.4 LAB induces MAPK and NF- $\kappa$ B activation in DC

Many studies have shown that TLR2 and TLR4 could sense the presence of microorganisms, which activate MAPK and NF- $\kappa$ B signaling to induce pro-inflammatory cytokine and chemokine production in DCs.<sup>29,30</sup> Thus, we analyzed the expression of TLR2, TLR4, and MyD88 in the jejunum and ileum of piglets from the CON and LAB groups. We observed that the LAB treatment enhances the expression of TLR2, TLR4, and MyD88 at the mRNA and protein levels in jejunum and ileum (Fig. 4A, B,

and Fig. S3A, B†). In line with these *in vivo* data from jejunum and ileum (Fig. 4A, B, and Fig. S3A, B†), we confirmed the higher expressions of TLR2, TLR4, and MyD88 at the protein levels in LAB-treated DC2.4 cells (Fig. 4C). To further explore the molecular mechanisms of LAB-mediated DC activation, we determined the expression of *Rela*, *AP1*, and *Irf3*, which are core transcript factors in NF- $\kappa$ B, MAPK, and IRF3 signaling pathways.<sup>32</sup> We found increased expression of *Rela*, *AP1*, *Irf3*, and *Trif* in jejunum and ileum in the LAB group (Fig. 4D and Fig. S3C†). These *in vivo* data indicated that LAB might induce NF- $\kappa$ B, MAPK, and IRF3 signaling activation to regulate intestinal immunity of piglets. In support of this, the LAB treatment, with either heat-killed or live form, significantly increased the expressions of p-ERK, p-JNK, p-p38, p-I $\kappa$ B $\alpha$ , and p-p65, but not p-IRF3 in DC2.4 cells (Fig. 4E and F). Collectively, these data suggested that LAB might induce DC activation through NF- $\kappa$ B and MAPK signaling to modulate intestinal immunity in suckling piglets.





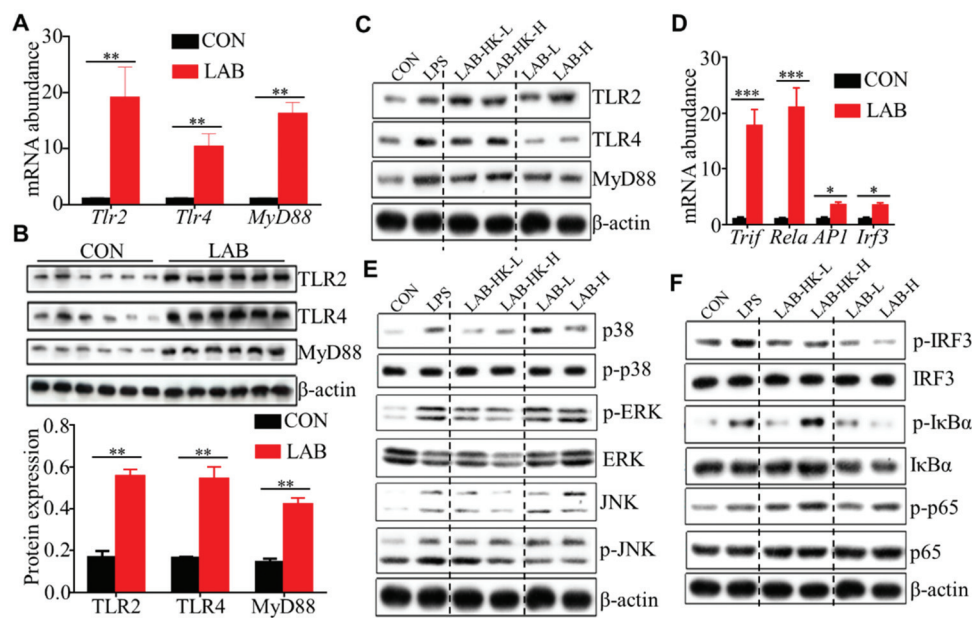
**Fig. 3** Oral administration of LAB induces DC maturation and activation. (A and B) The mRNA abundance of *CD80*, *CD86*, and *MHC-II* (A) and *Ccl20*, *Ccr6*, and *Cx3cr1* (B) in the ileum of piglets from the CON and LAB groups. (C) Protein levels of CCL20 and CX3CL1 in the serum of piglets. (D) Flow cytometry analysis of CD80, CD86, and MHC-II in DC2.4 cells stimulated with LPS, low dose of heat-killed (LAB-HK-L) and live LAB (LAB-L), and high dose of heat-killed (LAB-HK-H) and live LAB (LAB-H). (E) ELISA assay for IL-12, IL-2, TNF- $\alpha$ , and CCL20 in DC2.4 cells stimulated as in (D). Bar graphs show mean  $\pm$  SEM. N.S.  $P > 0.05$ , \* $P < 0.05$ , \*\* $P < 0.01$ .

### 3.5 LAB colonization in the suckling period exhibits long-lasting positive effects on intestinal immunity of weaned piglets

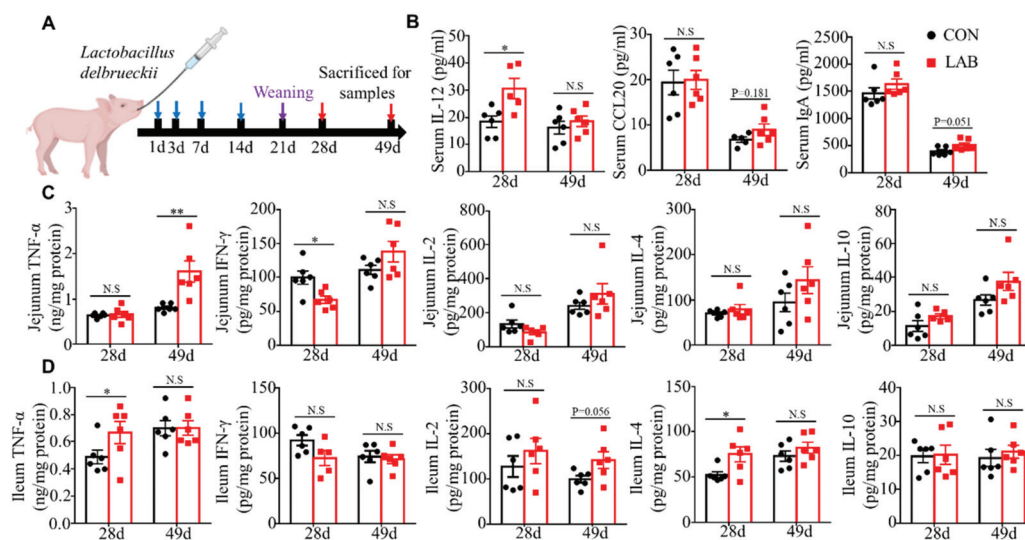
Early-life microbial colonization is the most important time for shaping intestinal and immune system development, which would exert long-lasting positive effects for enhancing the immune system and preventing the pathogenesis of several diseases.<sup>33,34</sup> Here, we further tested whether oral administration of LAB in suckling piglets could exert long-lasting effects on intestinal immunity after weaning (Fig. 5A). We observed that the LAB treatment in the suckling period enhanced the serum IL-12 levels on day 28, and had a tendency to increase the serum IgA levels on day 49 (Fig. 5B).

However, the LAB treatment failed to affect the serum IFN- $\gamma$ , IL-2, TNF- $\alpha$ , and CX3CL1 levels (Fig. S4†). Meanwhile, oral administration of LAB in the suckling period dramatically increased the protein levels of TNF- $\alpha$  in jejunum at day 49 and in ileum at day 28 (Fig. 5C and D). The protein levels of IL-4 in the ileum from the LAB group increased at day 28 (Fig. 5D). In contrast, the LAB treatment in the suckling period reduced the IFN- $\gamma$  protein levels in the jejunum (Fig. 5C). However, the protein levels of IL-10 and IL-2 in the jejunum and ileum at days 28 and 49 were comparable between these two groups (Fig. 5C and D). These data suggested that the oral gavage of LAB in suckling piglets could exert long-lasting effects on intestinal immunity after weaning.





**Fig. 4** LAB induces MAPK and NF- $\kappa$ B activation in DC. (A and B) The expression of TLR2, TLR4, and MyD88 at the mRNA (A) and protein (B) levels in the ileum of piglets from the CON and LAB groups. (C) Protein levels of TLR2, TLR4, and MyD88 in DC2.4 cells stimulated with LPS, low dose of heat-killed (LAB-HK-L) and live LAB (LAB-L), and high dose of heat-killed (LAB-HK-H) and live LAB (LAB-H). (D) The mRNA abundance of *Trif*, *Rela*, *AP1*, and *Irf3* in the ileum of piglets. (E and F) Immunoblot analysis of p-ERK, p-JNK, p-p38, p-I $\kappa$ B $\alpha$ , p-p65, and p-IRF3 in DC2.4 cells stimulated as in (C). Bar graphs show mean  $\pm$  SEM. N.S.  $P > 0.05$ , \* $P < 0.05$ , \*\* $P < 0.01$ .



**Fig. 5** LAB colonization in the suckling period exhibited long-lasting positive effects on intestinal immunity of the weaned piglet. (A) Schematic diagram of the experimental design. (B) Serum CCL20, IgA, and IL-12 levels in piglets from the CON and LAB groups. (C and D) Protein levels of TNF- $\alpha$ , IFN- $\gamma$ , IL-2, IL-10, and IL-4 in the jejunum (C) and ileum (D) of piglets. Bar graphs show mean  $\pm$  SEM. N.S.  $P > 0.05$ , \* $P < 0.05$ , \*\* $P < 0.01$ .

## 4 Discussion

Maintenance of gut homeostasis is a guarantee for achieving good performance of humans and animals. The immature development of the gut immune system during early life renders them more susceptible to food and microbial antigens.<sup>2,35</sup> Accumulating evidence has shown that early-life

microbial colonization is essential for gut homeostasis.<sup>6,26</sup> Myriads of probiotics including *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. contribute to gut immune system development under healthy or pathogenic conditions, which promote the growth performance of animals.<sup>5,9,12,36</sup> In line with previous studies, we found that oral administration of LAB increases the abundance of the





*Lactobacillus* genus and enhances intestinal immune responses in suckling piglets, which provide some evidence to support our previous observation that the LAB treatment improves the growth performance of piglets during the suckling and weaning period.<sup>19</sup> Of note, we observed that the LAB treatment increases IL-4 and IL-10 production in jejunum and ileum in suckling piglets. IL-10 and IL-4 are pivotal for the proliferation of B cells and differentiation of these cells into plasma cells, and are key factors that drive the secretion of IgA in the mucosal site.<sup>37–39</sup> In our previous studies, we found that the LAB treatment increases mucosal sIgA production in the jejunum and ileum from the suckling and weaned piglets.<sup>16,18</sup> Thus, we speculated that LAB-mediated mucosal sIgA secretion in the intestine is associated with its immunomodulation on IL-4 and IL-10 production, which need to be further investigated.

Intestinal DCs mediate tolerance to food antigens and gut commensal microbiota.<sup>30</sup> Maturation and differentiation of intestinal DCs can be regulated by many factors including colonization with *Lactobacillus* spp. or some other probiotic strains.<sup>40,41</sup> In this study, we found that LAB promotes DC maturation and activation, suggesting a possible link between LAB administration and improved intestinal immunity. We further found that LAB-induced DC maturation and activation are associated with TLR2/4-mediated NF- $\kappa$ B and MAPK signaling activation. It is well known that TLR-mediated NF- $\kappa$ B and MAPK signaling is critical to DC maturation and activation, and also crucial to cytokine production and co-stimulatory molecule expression.<sup>42–44</sup> Indeed, we observed that the LAB treatment increases the co-stimulatory molecules' (CD80 and CD86) expression and cytokines' (IL-12, TNF- $\alpha$ , and CCL20) production. In support of our observation, Elawadli *et al.* also found that the *Lactobacillus* treatment induces DC maturation and activation.<sup>45</sup> However, this is in disagreement with some other studies showing that probiotic treatment decreases maturation and inflammatory cytokine production of DCs after LPS or pathogen challenge.<sup>46,47</sup> Discrepancies of these results are possibly attributed to the different models used in our study because we evaluated the modulatory effects of LAB on DCs in a steady-state. Moreover, some investigations show that the probiotic treatment induces DCs to differentiate into different functional DC subsets, which provide signals to tune the innate and adaptive immune response.<sup>48,49</sup> However, whether oral administration of LAB could impact intestinal DC differentiation in suckling piglets remains to be studied.

Intriguingly, we observed that the LAB treatment increases the serum IL-12, jejunal and ileal TNF- $\alpha$  levels at days 28 and 49 in the weaned piglets. IL-12 and TNF- $\alpha$  are critical mediators for initiating the innate and adaptive immune response. Many studies have shown that TNF- $\alpha$  and IL-12 play important roles in enhancing the phagocytic and bactericidal effects of macrophages and neutrophils, promoting macrophage and DC activation, and inducing Th1 differentiation and cytotoxic T lymphocyte activation.<sup>50–53</sup> Thus, the increased IL-12 and TNF- $\alpha$  levels after weaning in the LAB-treated piglets imply that LAB exhibits long-term immunomodulatory effects

on intestinal immunity. Moreover, the administration of probiotics during the early life of humans or mice can prevent or treat diseases such as IBD and allergic diseases.<sup>54,55</sup> Some researchers ascribe the long-term implications to the effects of probiotics on increasing intestinal microbial diversity during early life.<sup>55,56</sup> In our study, we found that oral administration of LAB during the suckling period increases the abundance of *Lactobacillus* species, which would provide rational evidence for the enhanced intestinal immune response and growth performance in the weaning period.<sup>19</sup>

## 5 Conclusions

In summary, we found that oral administration of LAB in suckling piglets enhances intestinal immunity and induces DC maturation and activation. Notably, we found that the immunomodulatory properties of LAB during the suckling period exhibit long-term implications on intestinal immunity during the weaning period. Our data suggest that LAB can be developed as an immunoenhancing biological feed additive.

## Ethical statement

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Hunan Agricultural University and approved by the Animal Ethics Committee of Hunan Agricultural University (approval number: 20180722).

## Author contributions

Wei Peng and Xing-Guo Huang designed the experiment. Wei Peng, Ying-Hui Li, Shu-Ling Hou, and Ling-Yuan Yang conducted the experiments. Wei Peng, Li-Xiang Chen, and Jie-Lin Duan performed data analysis. Wei Peng, Guan Yang, Jie-Lin Duan, and Xing-Guo Huang wrote the manuscript.

## Conflicts of interest

The authors declared that there is no conflict of interest.

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