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Brief Communication

Oral administration of *Lactic acid bacteria* inhibits PEDV infection in young piglets

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ABSTRACT

Since the emergence of the highly pathogenic porcine epidemic diarrhea virus (PEDV) strain in 2010, the prevention of porcine epidemic diarrhea (PED) in pig farms remains problematic. To find the reasons behind the high mortality in young piglets, the relative mRNA expression of inflammation-related factors in infected pigs of different ages as well as uninfected pigs were detected by RT-qPCR. The results showed that the mRNA expression of these factors including IL-6 and TNF-α was more increased in infected younger piglets than infected older pigs. To clarify the relationship between these inflammation related factors, the pairwise linear correlation between the relative expression of these factors were analyzed and showed as network mapping with different correlation coefficients. A strong positive correlation was observed between the expression of various factors in 1-week-old piglets. Combined with the difference in mortality of PEDV infection in pigs of different ages, we hypothesized that *lactic acid bacteria (LAB)* could inhibit PEDV infection in newborn piglets, and an *in vivo* experiment was carried out. The results of survival rate and wet/dry ratio showed that *LAB* alleviated PEDV indued mortality and diarrhea. The detection of viral copies and tissue section staining showed less observed viruses in *LAB* treated pig. RT-qPCR results of gene expression in intestines showed that *LAB* modulated the gene expression of various host barrier genes, indicating that *LAB* is potential to inhibit PEDV infection by regulating the host intestinal barrier. However, to use *LAB* as therapy, how to improve the efficiency on inhibiting PEDV infection needs further studies.

1. Introduction

Since the highly pathogenic porcine epidemic diarrhea virus (PEDV) appeared in 2010, porcine epidemic diarrhea (PED) became a global threat to the pig industry (Li et al., 2020a). PEDV belongs to the family of alphacoronaviruses, and has 100% mortality in newborn piglets (Li et al., 2020b). PEDV is mainly transmitted through fecal and oral route, causing intestinal barrier damage, diarrhea and dehydration (Jung and Saif, 2015). The intestinal barrier includes mucus, epithelial cells and the underlying lamina propria (Salvo Romero et al., 2015). The epithelial layer also includes goblet cells which produce mucin 2, Paneth cells which produce lysozyme, and Microfold cells which express CK18 (Peterson and Artis, 2014; Soderholm and Pedicord, 2019). PEDV infection results in a host immune response in the intestines, including the production of IFNs, including type I IFN-β and type III IFN-λ, initiated by pattern recognition receptors (PRRs, e.g. NODs, TLRs), and inflammation-related cytokines (e.g. IL-6, IL-17, TNF-α) (Chen et al., 2020), as well as RPIK2 and IL-23 that were reported to be also involved in inflammation (Iwakura and Ishigame, 2006; McCarthy et al., 1998).

In our previous study, we demonstrated that PEDV infection reshapes the intestinal microbial distribution, and the strongest effects were observed for *Lactobacillus* and *Escherichia-shigella* (Yang et al., 2020). Both the absolute and relative abundance of *Lactobacillus* were increased while *Escherichia-shigella* were reduced in the intestine of PEDV infected 1-week pigs compared to uninfected pigs. However, the tendency of *Lactobacillus* and *Escherichia-shigella* in 2/4-week-old pigs' intestines is opposite, the absolute and relative abundances of *Lactobacillus* were reduced while *Escherichia-shigella* were increased in the intestine of

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Table 1
Detailed sequences of primers for RT-qPCR analysis.

Names	Forward primers	Reversed primers
Occludin	CAGGTGCACCCTCCAGATTG	ATGTCGTTGCTGGGTGCATA
IFN-γ	TGGTAGCTCTGGGAAACTGAATG	GGCTTTGCGCTGGATCTG
NOD1	ACTGACAGTGGGGTGAAGGT	TTTCCCAGTTTCAGGCACTTG
CK18	AGTTCTGTGGACAATGCCCG	CATCAATGACCTTGCGGAGC
Lysozyme	GGTCTATGATCGGTGCGAGT	AACTGCTTTGGGTGTCTTGC
PDB2	GCTGCTGACTGTCTGCCTCCTCTCT	CTGTTGAAGAGCGGGCAGGGGGAGA
Mucin2	GAGGAGAAGTGTGACGACCCCGA	CGGCGTGGGAGCACTGGCGGGAG
Caspase-3	TGGGATTGAGACGGACAGTG	CGCTGCACAAAGTGACTGGA
EGF	TCTGAACCCGGACGGATTTG	GACATCGCTCGCCAACGTAG
IL-1β	CAGCCAGTCTTCATTGTTCAGGTT	AGATTTGCAGCTGGATGCTC
IL-2	GTGAATATGATGATGAGACAGTAA	CAAGTCAGTGTTGAGTAGATG
IL-6	AATGCTCTTCACCTCTCC	TCACACTTCTCATACTTCTCA
IL-16	AATGCTCTTCACCTCTCC	TCACACTTCTCATACTTCTCA
IL-18	GTAGCTGAAAACGATGAAGACCTG	GGCATATCCTCAAACACGGC
IL-10	GTGAAGAGTGCCTTTAGC	TCTATGTAGTTGATGAAGATGTC
IL-22	GATGAGAGAGCGCTGCTACCTGG	GAAGGACGCCACCTCCTGCATGT
IFN-λ3	GTCCCTCTTGGAGGACTGGA	TGCTGTGCAGGGATGAGTTC
IFN-β	CGATACCAACAAAGGAGCAGCAA	CATCTCGTGGATAATCAATACTG
TLR2	TCACTTGTCTAACTTATCATCCTCTTG	TCAGCGAAGGTGTCATTATTGC
TLR3	AGTAAATGAATCACCCTGCCTAGCA	GCCGTTGACAAAACACATAAGGACT
TLR4	GCCATCGCTGCTAACATCATC	CTCATACTCAAAGATACACCATCGG
TGF-β1	GGAACCTGTATTGCTCTC	AATCATTGCTGTATTTCTGG
IL-17	AAGTCCAGGATGCCCAAA	CGGTTCAAGATGTTCAAGTTG
RIPK2	GTGGATGGGCACAAAATCCAG	TGGAAGCACTTTGCAACTTTGT
IL-23	CCTTCTCCGCCTCAAGATCC	TACTGGCTCAGAGTTGCTGC
TNF-α	GTCTCAAACCTCAGATAAG	GTTGTCTTTCAGCTTCAC
PEDV-M	GATACTTTGGCCTCTTGTGT	CACAACCGAATGCTATTGACG
IFN-λ1	GTCCCTCTTGGAGGACTGGA	TGCTGTGCAGGGATGAGTTC
GAPDH	CATCCATGACAACTTCGGCA	GCATGGACTGTGGTCATGAGTC

PEDV infected pigs. Combined with the difference in mortality of PEDV infection in pigs of different ages, we hypothesized that *lactic acid bacteria (LAB)* could inhibit PEDV infection in newborn piglets.

Lactic acid bacteria (*LAB*) has a strong anti-inflammatory effect (Saez-Lara et al., 2015), and some intestinal microbes are reported to affect viral infection (Lv et al., 2021). In this study, we demonstrated that PEDV infection induces the expression of many inflammatory cytokines in the intestine of 1-week-old piglets, and there is a strong interaction between these cytokines. To investigate whether *LAB* inhibits PEDV infection, an *in vivo* pigs' experiment was carried out. To provide more clues about the antiviral mechanism of *LAB*, the expression level of host immune factors and intestinal barrier related genes were examined. This study provides a new possibility for the prevention of PEDV, and more research needs to involve the use of *LAB* as a therapeutic intervention.

2. Materials and methods

2.1. RNA extraction and RT-qPCR

The intestines from uninfected and infected pigs of different ages were preserved in our lab. Total RNA from tissues was extracted using Trizol reagent (Invitrogen). Next, RNA was reverse transcribed into cDNA for the detection of specific genes by RT-qPCR (Vazyme-R223). The Probe qPCR SuperMix (Transtart) was used for viral copies detection. The ChamQ SYBR qPCR Master Mix (Vazyme) was used for the relative gene expression detection. The sequence of probe is 5′-FAM-TTC AGCATCCTTATGGCTTGCATC-TAMRA. The detailed sequences of primers are listed in Table 1. The detailed sample collection and experimental methods were listed in our previous study (Yang et al., 2020).

2.2. Viral and bacterial culture

The PEDV strain (GS-PEDV LJX) was generated and preserved in our lab. *LAB* strains including *Lactis lactococcus*, *Lactobacillus planturm* and *Lactobacillus parasei* were kind gifts of Dr. Junwei Ge from Northeast Agricultural University. To reach the logarithmic growth phase, *Lactis lactococcus* was cultured in GM17 medium for 4 h at 30 °C (Xiao et al., 2016), *Lactobacillus plantarum* and *Lactobacillus parasei* are cultured in MRS medium for 6 h at 37 °C. Bacteria were diluted and counted by plate counting at logarithmic stage (Mendonca et al., 2020). After counting, to collect the bacteria up to 10^9 CFU/strain, the bacteria were centrifuged (3000 rpm, 10min, 4 °C) and suspended with cold PBS. Then the three strains of bacteria were mixed with 0.5 mL PBS for oral administration in *vivo* experiment.

2.3. In vivo experiments

Nine PEDV-free newborn piglets from the same sows were purchased from a pig farm located in Dingxi, Gansu province and fed with a milk substitute from Bolai Yaoye Co.,Ltd. The gender of pigs was chosen randomly. The experimental setup is shown in Fig. 2A. Nine piglets were divided into three groups: control group (n = 3), infected group (n = 3), *LAB* group (n = 3) (10^9 CFU/each bacterial strain/time, 3 times a day). Piglets in the *LAB* group received *LAB* via oral application for seven days, while pigs in the other groups received PBS during this period. On day 7, animals in the infected and *LAB* groups were orally infected with PEDV (10^9 copies/pig), and health conditions were observed and recorded for 14 days. On day 21, all pigs were sacrificed and the intestines were collected for subsequent analysis. The diarrheal condition was recorded as follows (liquid, 3; semi-liquid, 2; semi-solid, 1; solid, 0). Fresh feces were collected and weighed (wet weight), and then weighed after drying (dry weight). The ratio between the wet weight and dry weight was the wet-dry ratio, which was used to evaluate the situation of diarrhea. For detecting the viral loads in feces, the anal fecal samples were collected every day and diluted with 1 mL PBS, 250 μL mixed samples were extracted for total RNA and detection. All experimental procedures and animal care protocols were approved by the guidelines for Care and Use of Laboratory Animals of Lanzhou Veterinary Research Institute (LVRI), Chinese Academy of Agricultural Sciences, China.

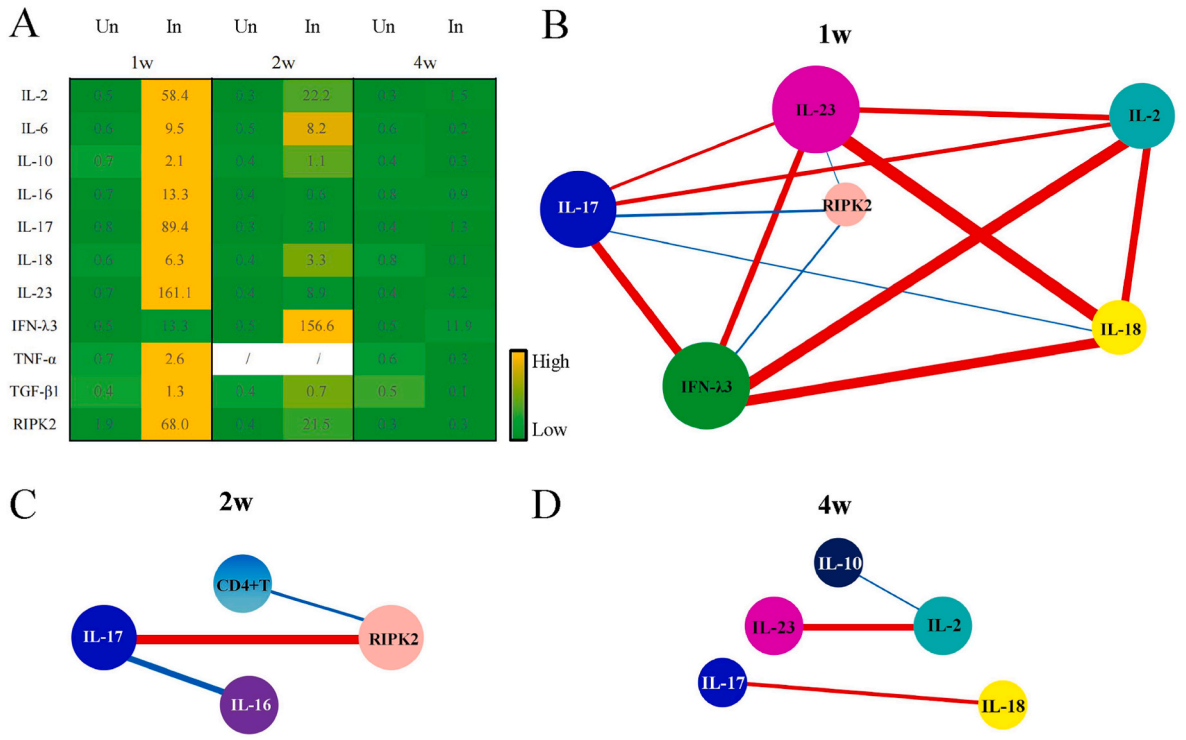

Fig. 1. PEDV induces strong inflammation-related factors expression in young piglets. RT-qPCR technique was utilized to detect mRNA expression levels of different cytokines in small intestine. (A) According to the $2^{\hat{}(-\Delta\Delta Ct)}$ calculation, based on the ΔCt of one replicate in the uninfected group, the relative changes of the remaining replicates in both groups were calculated, and then the average of the uninfected group or the infected group were calculated, presented as a heat map. The expression level of each molecule is regulated independently of the other. The higher the value, the yellower, and the lower the value, the greener. '/' means no Ct detected. The correlation networks between cytokines in pigs of 1-week (B). 2-Week (C) and 4-week (D) are shown. By linear correlation analysis, the R^2 values of the correlation are showed in sheet S1. The inflammation-related genes that did not show any correlation were excluded from the figure. According to R^2 values, values greater than 0.9 are linked as red lines, while values greater than 0.8 are linked as blue lines. The thicker the line, the stronger the correlation. The bigger the circle, the more connections the molecule has with other molecules.

2.4. Histological analysis and immunohistochemistry

The intestines were collected, fixed and dehydrated according to the standard protocol and then embedded in paraffin. The sections from tissues were deparaffinized in xylene, stained with Hematoxylin and Eosin and analyzed via optical microscope (Olympus, Japan) (Slaoui and Fiette, 2011). For the immunohistochemistry, the sections were renatured (microwave treatment 10min) to expose PEDV antigens and stained with a PEDV-N specific monoclonal antibody (IgG1κ, 1:200) made in our lab (Yang et al., 2020). The results were analyzed under an optical microscopy. For measuring the length of villi and crypt, nine villi from each section were randomly picked out and measured by ImageJ software. The ratios of villi and crypt were calculated and analyzed in GraphPad.

2.5. Data analysis

In pairwise linear correlation analysis of different cytokines, ΔCt is the data source of relative expression of each cytokine. After subtraction of interreference gene Ct in RT-qPCR result, ΔCt of each cytokine was acquired. The percentage of $CD4^+$ T cells in peripheral blood monocyte cells (PBMCs) was determined by flow cytometry analysis as reported (Grievink et al., 2016; Kreher et al., 2003). By pairwise linear correlation analysis between the ΔCt of all cytokines and percentage of $CD4^+$ T cells, the R^2 values of the correlation were acquired. All data and diagrams were analyzed and shown by GraphPad 7.0 and SPSS. The significant difference analysis was performed by one-way ANOVA ("***" means $p < 0.001$, "**" means $p < 0.01$, "*" means $p < 0.05$). Data were presented as Mean ± SEM.

3. Results

3.1. PEDV induces high expression of inflammation-related factors in young piglets

Previous work showed that the number of *Lactobacillus* was up-regulated in younger infected piglets, contrary to the tendency in older pigs. In order to further explore the reason behind this, RT-qPCR was utilized to detect the mRNA expression levels of inflammation-related cytokines in uninfected and infected pigs of different ages. As shown in the heatmap in Fig. 1A, expression levels of inflammation-related cytokines IL-2, IL-6, IL-16, IL-17, IL-18, IL-23 and RIPK2 were more than 10 times higher in 1-week-old piglets compared to levels in uninfected piglets. Compared to levels in uninfected pigs of 2-week-old pigs , the expression levels of IL-2, IL-6, IL-23, RIPK2 were more than 10 times higher. The increased levels were lower in 2-week-old pigs compared to the increased levels in infected 1-week-old pigs. In 4-week-old infected pigs, only the expression levels of IL-2, IL-17 and IL-23 showed a tendency to increase (less than 10 times) compared to uninfected pigs. Interestingly, when comparing pigs of different ages, the levels of IFN-λ3 were 10 times higher in infected 2-week-old pigs compared to uninfected pigs. Next, linear correlations between relative expression levels of these cytokines and the percentages of $CD4^+$ T cells in pigs of different ages (including uninfected and infected) were analyzed. An overview of all data and R^2 values is shown in supplemental material sheet S1. According to the R^2 values, networks were generated as shown in Fig. 1B, C and 1D. Inflammation-related genes that did not show any correlation with $CD4^+$ T cells were excluded from the figure. In 1-week-old pigs, IL-23, IL-18, IFN-λ3, IL-2, IL-17 showed a strongly positive linear correlation with each other, followed by the

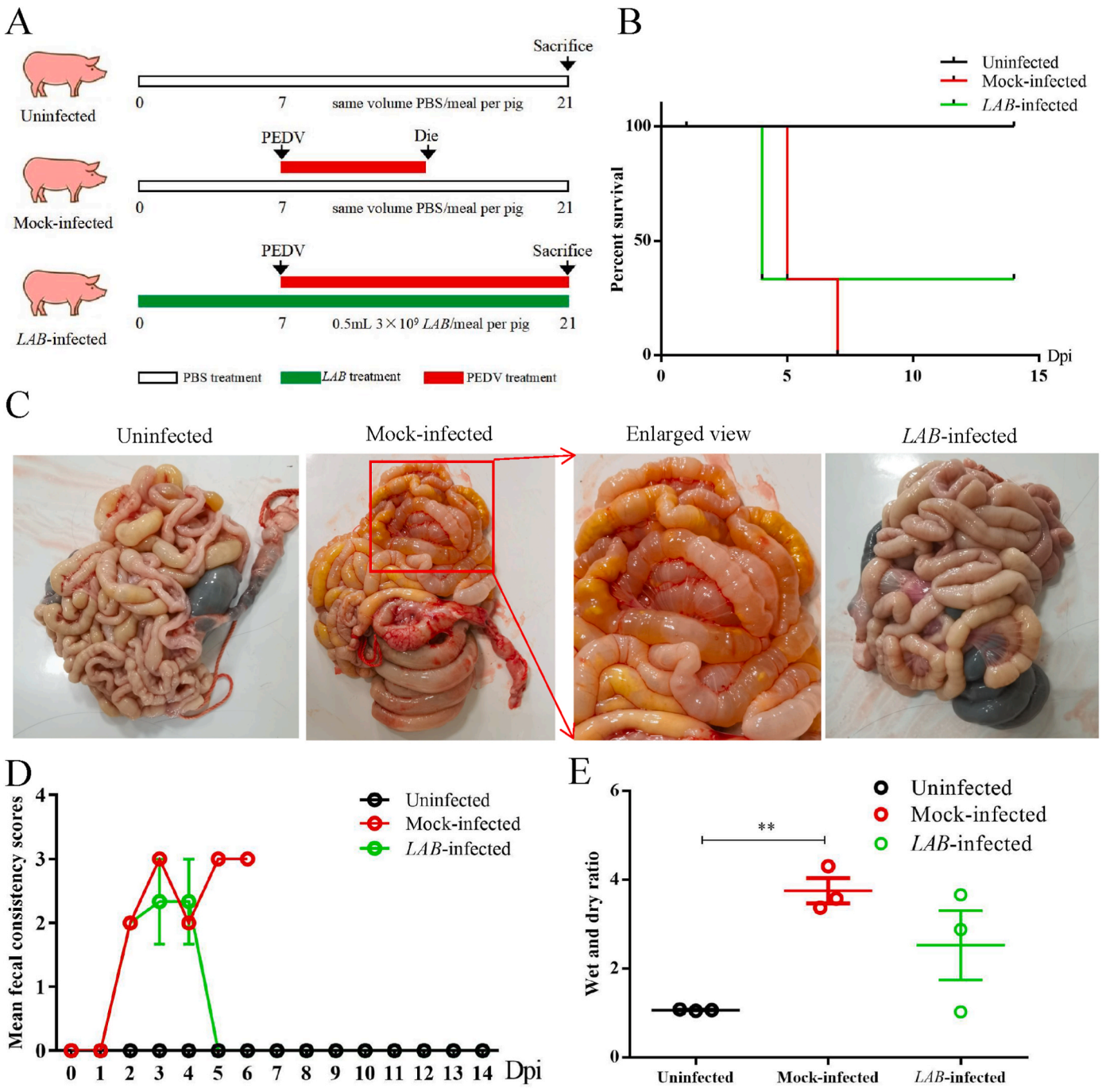

Fig. 2. *LAB* are potential to inhibit PEDV infection in piglets. (A) The flow chart of the *in vivo* experimental setup is shown. (B) Survival curve of pigs in different groups are shown with three pigs per group. (C) The clinical anatomy changes of intestines in pigs from different groups were calculated and shown. (D) The fecal consistency scores in pigs of different groups were recorded. (E) The ratio between wet and dry feces in pigs from different groups was calculated and shown.

correlation with RIPK2. In 2-week-old pigs, only IL-17 and RIPK2 were strongly positively correlated, followed by the correlation with IL-16 and $CD4^+$ T cells. The relative expression of RIPK2 positively correlated with the percentage of $CD4^+$T cells in PBMCs. In 4-week-old pigs, only IL-2 and IL-23, IL-17 and IL-18 had a strong positive correlation with each other, while the correlation of IL-10 and IL-2 was only weak. To summarize, 1-week-old piglets that are infected with PEDV show enhanced expression of inflammation-related cytokines, and there is a strong positive correlation among these individual cytokines, while this positive correlation in the older pigs is much weaken.

3.2. *LAB are potential to inhibit PEDV infection in piglets*

To test whether anti-inflammatory cytokine profiles induced by the *LAB* bacteria alleviate the development of symptoms and prevent PEDV infection in 1-week piglets, *LAB* was orally administrated to 1 week old piglets prior to PEDV infection (Fig. 2A). As shown in the survival curve in the *LAB* group one piglet survived (Fig. 2B), while in the infected group none of the piglets survived. Comparing to the uninfected pigs' intestine, the intestinal wall of infected pigs was thinner and the guts were full of yellow liquid (Fig. 2C). In the cured pig, *LAB* restores the intestinal physical condition as usual. In addition, the improved fecal consistency scores and decreased wet-dry ratio in pigs of the *LAB* group,

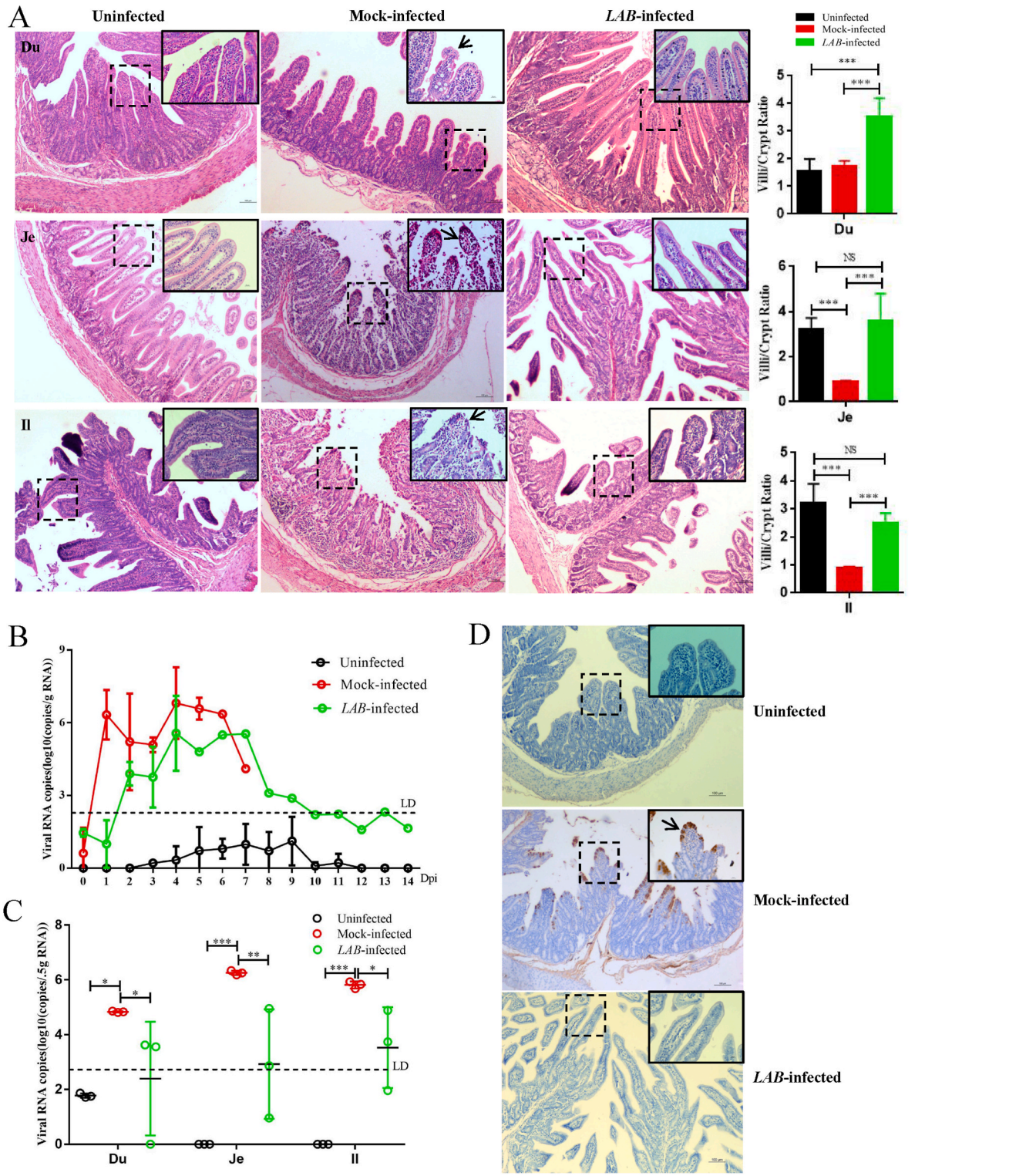

Fig. 3. *LAB* are able to delay viral infection and reduce viral loads

(A) HE staining was used to investigate the possible pathological changes in the intestines. The scale shown is 100 μm, and the arrows points to representative damaged or infected epithelial cells in each figure. The dashed boxes were the located view of magnification, and the enlarged views were showed in the top right corner. (B) The viral shedding in the daily feces was detected by real-time qPCR. (C) The viral loads in the intestine of pigs from different ages was detected by real-time qPCR. (D) Immune-histochemical staining (PEDV-N protein) was used to investigate the viral presence in the intestines. The scale shown is 100 μm, and the arrows points to representative damaged or infected epithelial cells in each figure. The dashed boxes were the located view of magnification, and the enlarged views were showed in the top right corner.

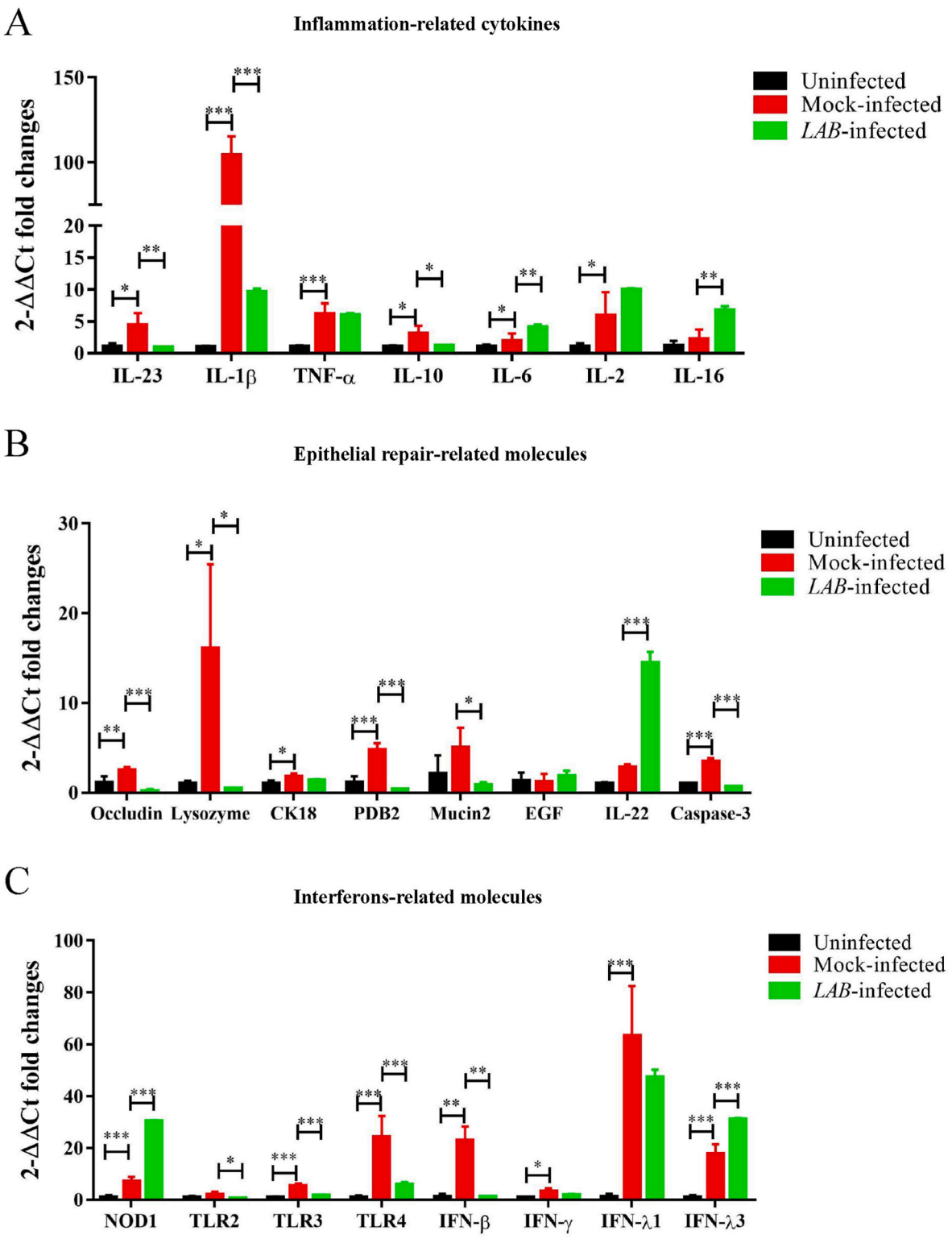

Fig. 4. *LAB* interfere with the expression of intestinal barrier related molecules in infected pigs. The mRNA expression level of inflammation-related genes is shown in (A). The mRNA expression level of intestinal barrier related molecules is shown in (B). The mRNA expression level of pattern recognition receptors and IFNs is shown in (C). Values represent MEAN ± SEM (n = 3).

suggesting that the diarrheal situation was improved, and the absence of symptoms of diarrhea were observed in the surviving pig in the *LAB* group (Fig. 2D and E).

3.3. *LAB* are able to delay viral infection and reduce viral loads

To further explore the intestinal villi condition, HE staining of villi was shown in Fig. 3A. Results showed that no epithelial cells abscission, villi fracture and decreased villi-crypt ratio were observed in the cured pig treated with *LAB* while these effects were readily detected in the

infected group (Fig. 3A). Detection of viral shedding in feces indicated that virus infection in *LAB* group was delayed by one day compared to the infected group, the virus titer was lower from day 7 onwards and recovered from day 10 onwards in the *LAB* cured pig compared to infected group (Fig. 3B). Analysis of the viral loads in the intestines showed that less virus was detected in *LAB*-infected pigs and no virus was present in duodenum, jejunum and ileum of the *LAB* cured pig (Fig. 3C). In addition, histochemical analysis showed no positive staining for the PEDV-N protein in epithelial cells in the LAB treated pigs (Fig. 3D). Collectively, *LAB* treatment effectively delayed viral infection and decreased viral loads in piglets, and the intestinal villi of the *LAB* cured pig is as healthy as in the uninfected group.

3.4. *LAB interfere with the expression of intestinal barrier-related molecules in infected pigs*

To explore a possible mechanism that may explain the finding in the *LAB* treated pig, RT-qPCR was used to detect mRNA levels of inflammation-related genes at Day 21 (Fig. 4A). Interestingly, mRNA expression levels of IL-23, IL-1β and IL-10 in the intestine of the *LAB* group were significantly lower compared to levels in the infected group. The expression level of TNF-α in *LAB* group was similar to that in infection group, but expression levels of IL-6, IL-2 and IL-16 tended to be up-regulated. In addition, mRNA expression levels of several intestinal barrier related molecules were measured (Fig. 4B). The *LAB* treated pig showed lower mRNA expression levels of occludin, lysozyme, PDB2, mucin2, caspase-3 but higher levels of IL-22 compared to untreated infected pigs. No differences in mRNA expression levels of CK18 and EGF were observed in intestines. Furthermore, mRNA expression levels of PRRs and IFNs were detected by RT-qPCR (Fig. 4C). Interestingly, the mRNA expression levels of TLR2, TLR3, TLR4, IFN-β were significantly decreased compared to the infected group while the mRNA expression levels of IFN-γ and IFN-λ1 were unchanged. The mRNA expression levels of NOD1 and IFN-λ3 were significantly increased in the *LAB* treated pig compared to the infected pigs.

4. Discussion

In this study, the effect of oral administration of anti-inflammatory bacteria like *LAB* on the outcome of a PEDV infection in newborn piglets was investigated. In 1-week-old piglets, PEDV infection induces the highest expression level of inflammation-related cytokines, and there is a strong positive correlation between theses cytokines compared to that in older pigs. The high level of inflammation-related cytokines aggravate tissue damage and can lead to high morbidity and mortality in young piglets (Chen et al., 2018). Furthermore, Peyer's' patches can be observed clearly in 2-week-old or older pigs but not yet in 1-week-old piglets, suggesting the gut-associated lymphoid tissues of young piglets is less well developed (Barszcz and Skomiał, 2011). Increased expression of RIPK2, IL-17 after infection and their correlated relationship with the percentage of $CD4^{+}$T cells in 2-week-old pigs, may indicate that pigs more than 2-week-old might start to regulate adaptive immunity to control the development of disease by affecting the subpopulations of $CD4^{+}$T cells (Subramaniam et al., 2017). Therefore, we speculate that initiating adaptive immunity is difficult for the prevention after viral infection in 1-week-old piglets. According to the results in previous study, the higher inflammatory cytokines are possibly caused by high amount of PEDV infection in 1-week piglets. Comparing to 2-week or 4-week piglets, 1-week piglets have higher viral loads and less LAB, moreover, PEDV infection increase the abundance of *Lactobacillus* (Yang et al., 2020, 2022). Therefore, we speculated that *Lactobacillus* is involved in protecting piglets and inhibiting intestinal injury via modulating innate immunity or increasing the intestinal barrier and its function.

The results of the *in vivo* experiment demonstrate that *LAB* delay PEDV infection and reduce viral loads in intestines, positively improving the intestinal health of infected piglets. First of all, in the *LAB* treated group lower levels of inflammatory molecules including IL-23, IL-1β were observed which was paralleled by less inflammatory damage as reflected in the physical condition of the intestine. Upregulation of IL-2, IL-6, and IL-16 has been reported to be involved in T cell development and stimulation of antibody production, suggesting that *LAB* also induces the development of adaptive immune cells in piglet's intestines (Boyman and Sprent, 2012; Richmond et al., 2014; Tanaka et al., 2014). Secondly, the expression levels of genes related to functional epithelial cells were restored to the initial level via *LAB* treatment. IL-22, as an important repair cytokine for intestinal injury and epithelial cell regeneration (Lindemans et al., 2015), was significantly up-regulated upon *LAB* treatment. These results suggest a potential role for *LAB* in strengthening the intestinal barrier as well as the repair of epithelial damage upon PEDV infection. Finally, the levels of NOD1 and IFN-λ3 were higher in the *LAB* treated pig that survived PEDV infection. This opens the possibility that *LAB* may exert its antiviral effect also at the innate level by inducing IFN-λ3, which is in agreement to an earlier study by Liu et al. (Li et al., 2019), which reported that porcine IFN-λ more efficiently curtails the PEDV infection than type I IFN-α/β, with stronger effects of IFN-λ3 compared to IFN-λ1.

To conclude, in the *LAB* treated group, one out of three piglets was showed in recovered condition, suggesting that *LAB* may have potential to the prevent PEDV. However, to improve the efficiency of *LAB* on the prevention of PEDV infection, the species and numbers of *LAB* taken orally should be optimized. Therefore, more research is still needed into the mechanism how *LAB* prevents PEDV infection.

5. Conclusion

In summary, we show that oral administration of *LAB* is potential to inhibit PEDV infection in pigs, and restore the damaged intestinal villi to healthy condition, causing no diarrhea in the clinical symptoms comparing to the infected pigs. Besides, the inflammatory responses in *LAB* treated pig are inhibited, intestinal barrier are strengthened and type III interferon levels are significantly upregulated. These data suggest a potential role for *LAB* in the prevention of PEDV infection in pigs.

Credit author statement

Shanshan Yang: conceived this paper, designed and conducted the experiments, analyzed the data, and Writing – original draft. Shuxian Li: contributed with the paraffin section staining. Yabin Lu: contributed with the cDNA preparation of tissues. Christine A. Jansen: contributed to discussions and revised the manuscript. Huub F.J. Savelkoul: contributed to discussions and revised the manuscript. Guangliang Liu: conceived this paper, contributed to discussions and revised the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2022.12.005.

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