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In vivo evaluation of preventive effect of *Lactobacillus reuteri* on porcine epidemic diarrhea in suckling piglets

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Abstract : Lactic acid bacteria have been reported their beneficial roles on host including reduction of infectious diarrhea problems. In this study, preventive effect of *Lactobacillus* (*L.*) *reuteri* HY25101 and *L. johnsonii* HY25103 on porcine epidemic diarrhea virus (PEDV) was investigated in suckling piglets. Two groups of one day old PEDV naïve piglets were orally administered *L. reuteri* HY25101 and *L. johnsonii* HY25103 for three days respectively before challenge with lethal dose of PEDV. In second experiment, passive immunized one day old piglets using colostrums containing PEDV specific IgA were used. The survival rates of the *L. reuteri* HY25101 administered group were significantly higher than that of *L. johnsonii* HY25103 administered group and viral shedding was rapidly diminished in *L. reuteri* HY25101 administered group. Interestingly piglets born from the sow immunized with attenuated PEDV vaccine were not completely protected from PEDV challenge, however coadministeration of *L. reuteri* HY25101 and colostrums containing PEDV specific IgA were more effectively prevent PEDV infection. These results suggested that dietary treatment using *L. reuteri* HY25101 could reduce diarrheal problem and mortality rate caused by PEDV in suckling pigs. In addition, *L. reuteri* HY25101 could be used as one of effective compensation treatment with attenuated live vaccine for PED.

Keywords : lactic acid bacteria, *Lactobacillus johnsonii*, *Lactobacillus reuteri*, porcine epidemic diarrhea, probiotic

Introduction

Lactic acid bacteria (LAB) played an important role as probiotics in balance of intestinal microflora, reinforcement of microbial barrier in digestive tract and prevention of colonization of pathogenic microorganism. Additionally LAB increased mucosal secretory IgA and IgM responses and also improved non-specific immunity such as phagocytosis and cytokine production [4, 8]. *Lactobacillus* (*L.*) *reuteri* was primarily isolated from human breast milk [7]. *L. reuteri* strain SD2112 showed therapeutic effect on rotavirus-related diarrhea in clinical trials [12, 13]. Another Gram-positive bacteria, *L. johnsonii*, was primarily isolated from feces of human and animals [2]. Some strains of *L. johnsonii* showed therapeutic effect on *H. pylori* infection [1, 9]. Porcine epidemic diarrhea (PED) is one of the major contagious enteric diseases in swine industry. The porcine epidemic diarrhea virus (PEDV) is member of the genus Coronavirus, family Coronaviridae, enveloped, single-stranded RNA virus. The obvious clinical sign of PED is watery diarrhea with anorexia, vomiting and dehydration. In previous research, two species of LAB isolated from swine feces showed *in vitro* antiviral activity against PEDV. Two isolates were identified as *L. reuteri* and *L. johnsonii* by alignment of nucleotide sequence of the 16S rRNA (unpublished data). There has been no investigation of preventive effect of

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probiotics on PED in suckling piglets. The aim of this study was to evaluate preventive effect of dietary *L. reuteri* HY25101 and *L. johnsonii* HY25103 on virulent PEDV in suckling piglets.

Materials and Methods

Bacterial strains and growth condition

L. reuteri HY25101 and *L. johnsonii* HY25103 were originally isolated from swine feces in Cheonan, Korea. These bacteria were cultured in MRS broth (Difco, USA) at 37°C and colony morphological characterization and colony forming units (CFU) of each bacterium was confirmed on MRS agar plate.

Virulent virus preparation

Highly virulent field isolate of PEDV was propagated by passage in one day old piglet, which was seronegative for PEDV. The animal was euthanized in acute phase of diarrhea and small intestine was collected. The small intestine was homogenized within PBS in a 1 : 20 (w/v) ratio. Two groups (seven piglets/group) were orally inoculated 10^{-3} and 10^{-4} diluted the homogenate respectively. Viral titer was calculated using the Reed-Muench method.

RT-PCR of PEDV

RT-PCR was performed to determine viral shedding rate of fecal samples from PEDV challenged piglets. Viral RNA was extracted from 300 µl of each fecal sample using the RNeasy mini kit (Qiagen, Netherland) according to the manufacturer's instructions. Following primer pair was employed for amplification of the partial S gene of PEDV: 5'-ACAAGTCTCGTAACCAGTCC-3' (forward), and 5'-GTATCACCACCATCAACAGC-3' (reverse). One-step RT-PCR procedure was performed using *Maxime* RT-PCR premix Kit (iNtRON Biotechnology, Korea) in a thermocycler (T-personal; Biometra, Germany) according to the manufacturer's instruction with annealing condition at 50°C for 45 sec. Nested PCR was performed to determine concentration of PEDV field isolate. 1 µl of each first round PCR amplification product was used as template with internal primer pair, 5'-CAAGAGCAGGAACCAGTCAA-3' (forward) and 5'-TCACGAACAGCCACATTACC-3' (reverse). 25 µl of PCR mixture contained 2.5 µl of 10X buffer (10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$), 0.2 mM each dNTPs and 0.5 µl of 5 U/µl rTaq DNA polymerase (Elpisbio, Korea). The amplification was performed at 94°C for 5 min, followed by 35 cycles of 95°C for 45 sec, 55°C for 45 sec and 72°C for 1 min.

in vivo investigation of preventive effect of LAB on PEDV

in vivo experiments were designed as Table 1. Two littermates of one day old crossbred piglets were purchased from a PEDV free herd. In Exp 1, piglets in group I (n = 8) were orally administered with 10^{10} CFU of *L. reuteri* HY25101/piglet for three days and group II (n = 13) were orally administered with 10^{10} CFU of *L. johnsonii* HY25103/piglet for three days. Piglets in group III (n = 8) born from a PEDV vaccinated sow with 1 ml of attenuated PEDV strain DR13 [15] were inoculated with 2 ml of PBS for three days. After inoculation, all groups were challenged with lethal dose of 10^{-2} diluted virulent PEDV. Degree of diarrhea and viral shedding via fecal route were observed daily. All death piglets were autopsied for examination of pathological changes. In Exp 2, to

Table 1. *In vivo* experimental designs

	Group	Treatments			
		Lactobacillus reuteri	Lactobacillus johnsonii	Vaccine*	S2A†
Exp 1	I	√	–	–	–
	II	–	√	–	–
	III	–	–	√	–
Exp 2	I	√	–	–	√
	II	–	√	–	√

*The modified live porcine epidemic diarrhea vaccine (Green Cross veterinary products Co., Korea) was used to immunize pregnant sow.
†The recombinant yeast expressing S2A antigen of porcine epidemic diarrhea virus was used to immunize pregnant sows.

induce passive immunity of piglets, 50 ml of recombinant yeast (10^9 CFU/ml; Korea Yakult R&D Center, Korea) expressing S2A epitope of PEDV was orally inoculated into two pregnant sows at 5 weeks and 3 weeks before delivery. Two littermate piglets born from the sows were used in Exp2. Piglets of group I (n = 11) were orally administered with 10^{10} CFU of *L. reuteri* HY25101/piglet and colostrums containing PEDV specific IgA for four days and group II (n = 9) was orally administered with 10^{10} CFU of *L. johnsonii* HY25103/piglet and the colostrums for four days before lethal dose of 10^{-2} diluted virulent PEDV challenge. Degree of diarrhea and viral shedding via fecal route were observed. All death piglets were autopsied for examination of pathological changes.

Measurement of PEDV specific antibody by ELISA

In Exp 2, S2A specific antibodiy levels in sera and colostrums from the sows immunized using the recombinant yeast was analyzed by ELISA. Serum samples from the sows were collected at 4 weeks and 2 weeks before delivery. Colostrums were collected immediately after delivery. S2A antigen was diluted with 0.05 M carbonate-bicarbonate buffer (pH 9.6) in a 1 : 1,000 ratio and 96-well microplates were coated with the diluted antigen at 4°C for overnight. After three times washing with PBST, the plates were blocked with PBS containing 0.1% BSA (Sigma, USA) at 37°C for 1 h. Subsequently, the plates were washed three times with PBST and 80 μl of each test sera was added into each well. The Plates were then incubated at 37°C for 1 h. After washing, 90 μl of 1 : 1,000 diluted horseradish peroxidase conjugated goat anti-pig IgG (Komabiotech, Korea) was added into each well and incubated at 37°C for 1 h. The plates were developed with commercial substrate, OPD (o-phenylenediamine; Sigma, USA) without light exposure. After 15 min, the reaction was stopped using 1 M H_2SO_4. Optical density of solution was measured at 490 nm ($O.D_{490}$). S2A specific IgA level of the colostrums were assayed by ELISA as same as above. 1 : 1,000 diluted HRP conjugated goat anti-pig IgA (Komabiotech, Korea) was used for secondary antibody.

Histopathological analysis

For standard histochemical staining, paraffin-embedded tissues were sectioned (6 μm), deparaffinized with xylene (Merck, USA), and rehydrated in graded ethanol solutions. The tissues were stained with Gill's hematoxylin (Merck, USA) and eosin (Merck, USA) and cleared in xylene (Merck, USA). Histopathologic changes were analyzed using an Eclipse TE2000-U Microscope (Nikon, Japan).

Statistical analysis

Statistical analysis of survival rate of each groups were performed by Fisher's Exact test. The SAS 9.1 software package (SAS Institute, USA) was used for statistical tests.

Results

Determination of virulent PEDV titer

The propagated virulent PEDV field isolate was serially ten-fold diluted and the presence of PEDV was tested by nested PCR. In first round PCR, expected size of PCR product was able to be detected until the 10^{-2} dilution (Fig. 1 lanes 1-3) and the smaller PCR product was able to be detected until the 10^{-5} dilution in second round PCR (Fig. 1 lanes 1-6). To determine LD_{50} of the propagated PEDV, two groups (seven pigs/group) were inoculated with 10^{-3} and 10^{-4} diluted the

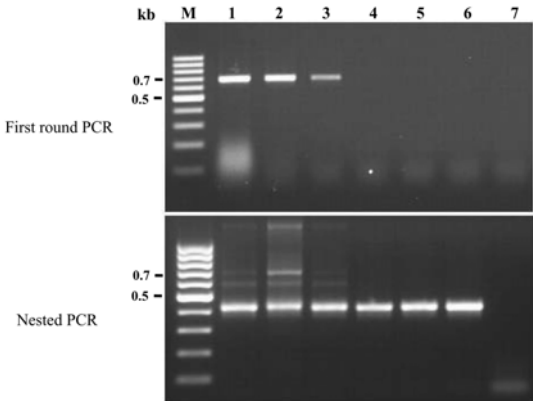

Fig. 1. Determination of concentration of the propagated virulent porcine epidemic diarrhea virus (PEDV). PEDV derived from small intestines of the suckling piglet orally inoculated with field isolate was serially 10-fold diluted. Amplification products obtained by first and second round PCR were separated using agarose gel electrophoresis. Lane M, DNA molecular weight marker and lanes 1-7 indicate 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} diluted PEDV samples.

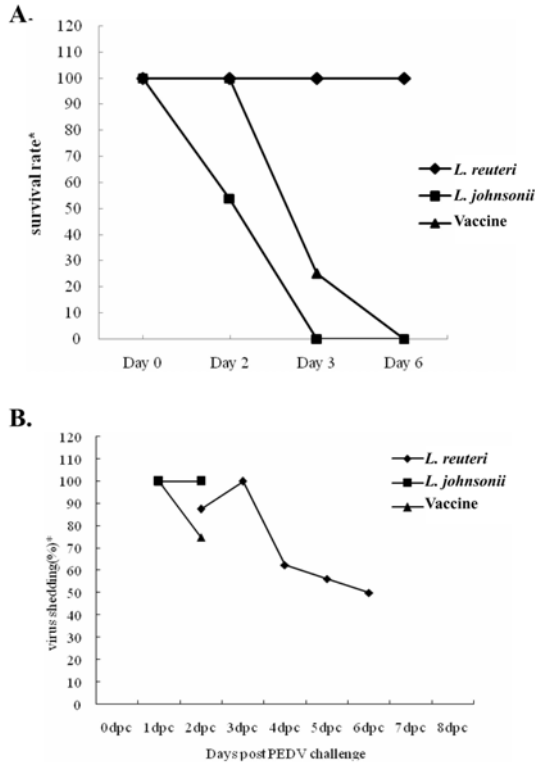

Fig. 2. Survival rate and viral shedding rate in Exp 1. (A) Survival rates of all groups were checked at 0, 2, 3 and 6 dpc. *Survival rate: Nnumber of live piglets/Number of total challenged piglets. Statistical analysis of survival rate of each groups was performed by Fisher's Exact test (p = 0.007). (B) Viral RNA was extracted from fecal swab samples and the partial S gene of PEDV was amplified. The viral shedding of group II and III were not determined from 3 dpc, because most of piglets in both groups died at 3 dpc. *Virus shedding: positive fecal swabs/ Total fecal swabs of live piglets.

propagated PEDV. Three piglets survived in the 10^{-4} diluted virulent PEDV inoculated group, while all seven piglets inoculated 10^{-3} diluted virulent PEDV died. The virulent PEDV titer was determined approximately $10^{4.3}LD_{50}$/ml. 10^{-2} dilution of virulent PEDV was decided as challenge dose.

***in vivo* evaluation of the preventive effect of *L. reuteri* HY25101 and *L. johnsonii* HY25103 on PED**

In Exp. 1, all piglets developed clinical signs after virulent PEDV challenge. The clinical signs were diarrhea, anorexia, vomiting, lying down and lethargy. All 8 piglets of *L. reuteri* HY25101 administered group I survived until 6 days post challenge (dpc) and this survival rate was significantly higher than those of other groups. The onset of diarrhea and viral shedding of group I started at 2 dpc and viral shedding rate of group I decreased to 50% at 6 dpc. Virus shedding rate of *L. johnsonii* HY25103 administered group II was 100% at 1 and 2 dpc and all piglets died by 6 dpc. In case of passive vaccinated group III, virus shedding rate decreased to 70% at 2 dpc, but all piglets died by 6 dpc (Fig. 2). Formalin fixed intestinal tissues of piglets were analyzed for histopathological alterations of PEDV. In group I, exfoliation of enterocytes and unstainable enterocytes on the villous tips were observed (Fig. 3a), although piglets survived until 6 dpc. In group II and group III, the villi were collapsed and unstained due to epithelial cell death and terminal web disappearance (Figs.3b and c). These results suggested that *L. reuteri* HY25101 inoculation offered more effective protection on PEDV than *L. johnsonii* HY25103 inoculation.

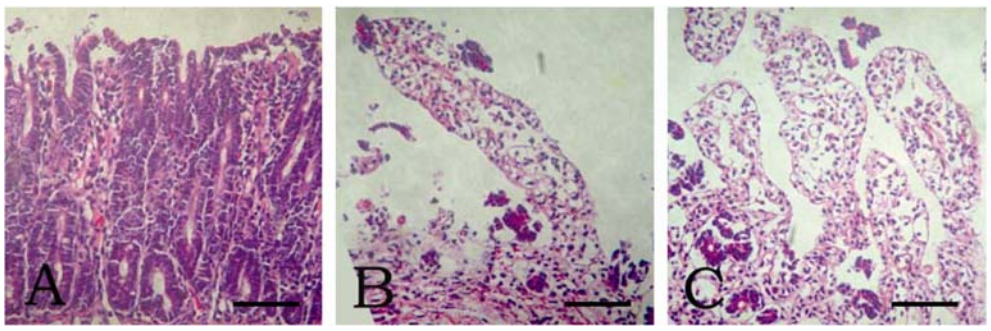

Fig. 3. Jejunum from lactobacillus inoculated piglets after challenging with virulent PEDV. Intestines were collected immediately from dead piglets or euthanized piglets at 6 dpc. (A) *Lactobacillus reuteri* HY25101 administered group (B) *Lactobacillus johnsonii* HY25103 administered group (C) The PEDV passive vaccinated group. H&E stain. Bar = 100 μm.

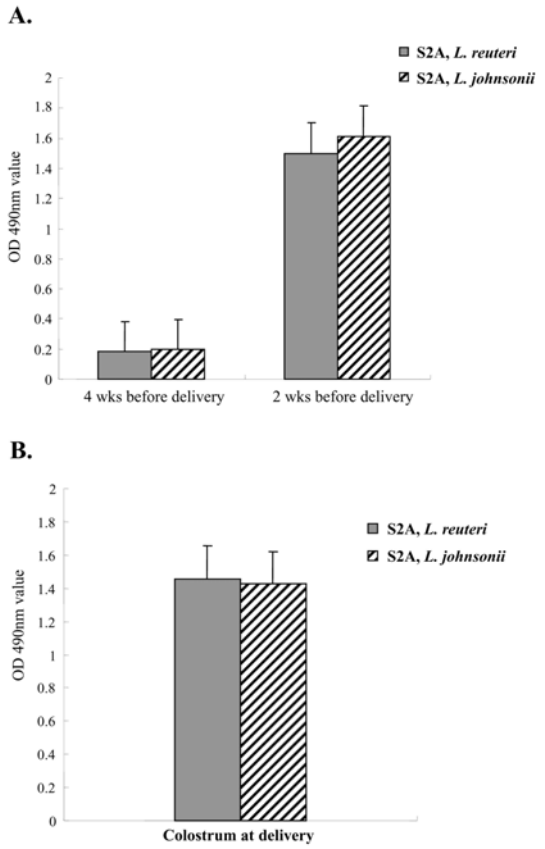

Fig. 4. S2A epitope specific antibody responses of serum samples and colostrums from the pregnant sows against. Two sows were orally inoculated twice with the yeast expressing S2A epitope of PEDV at 5 and 3 weeks before delivery. The ELISA plates coated with purified S2A protein were used to measure the specific antibody level. (A) Serum samples were collected at 4 weeks and 2 weeks before delivery. (B) Right after delivery colostrums were collected.

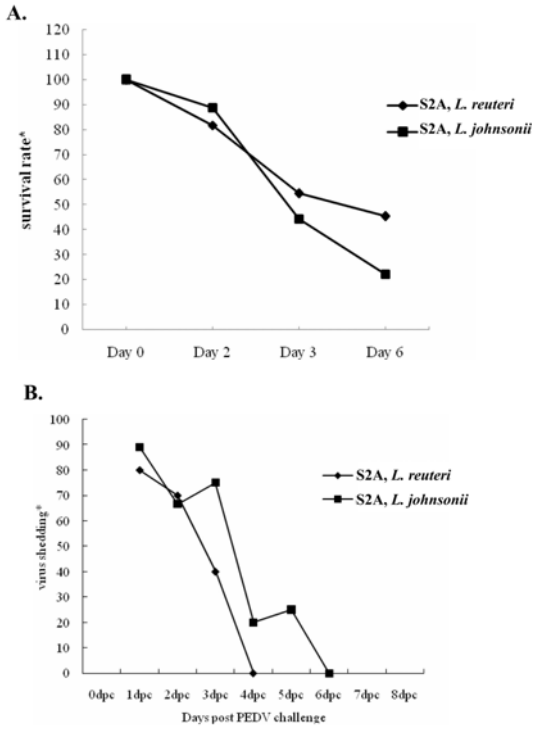

Fig. 5. Survival rate and viral shedding rate in Exp 2. (A) Survival rates of all groups were checked at 0, 2, 3 and 6 dpc. *Survival rate: Number of live piglets/Number of total challenged piglets. (B) Viral RNA was extracted from fecal swab samples and the partial S gene of PEDV was amplified. *Virus shedding: Positive fecal swabs/Total fecal swabs of live piglets.

***in vivo* evaluation of the preventive effect of *L. reuteri* HY25101 and *L. johnsonii* HY25103 on PED in PEDV passive immunized piglets**

PEDV S2A antigen specific IgG level of sera from the pregnant sows was considerably increased after second inoculation (Fig. 4a). Antigen specific IgA level of colostrums from corresponding sows were also significantly increased (Fig. 4b). These results suggested that the colostrums, inoculated into the piglets of both groups, contained PEDV specific IgA. All piglets developed clinical signs after PEDV challenge. Survival rates of both groups were checked at 0, 2, 3 and 6 dpc. *L. reuteri* HY25101 inoculated group I showed higher survival rate than that of *L. johnsonii* HY25103 inoculated group II, however these results didn't show any significant difference in statistical analysis (Fig. 5a). Viral shedding appeared at 1 dpc in both groups. Viral shedding rate of group I was decreased faster than that of group II. Viral shedding of group I was ceased at 4 dpc, but that of group II was ceased at 6 dpc (Fig. 5b). The jejunal enterocytes of survival piglets were clearly polarized and nuclei were localized at the base of enterocytes (Figs. 6a and c). The typical histopathologic lesions of PED were observed in the small intestinal sample of dead piglets. Markedly shortened villi of the jejunum and enlarged vacuoles appeared in the enterocytes lining most of villous surface of the small intestines (Figs. 6b and d). These results suggested that the administration of *L. reuteri* HY25101 into the piglets possessing passive immunity for PEDV offered more effective protection than administration of *L. johnsonii* HY25103.

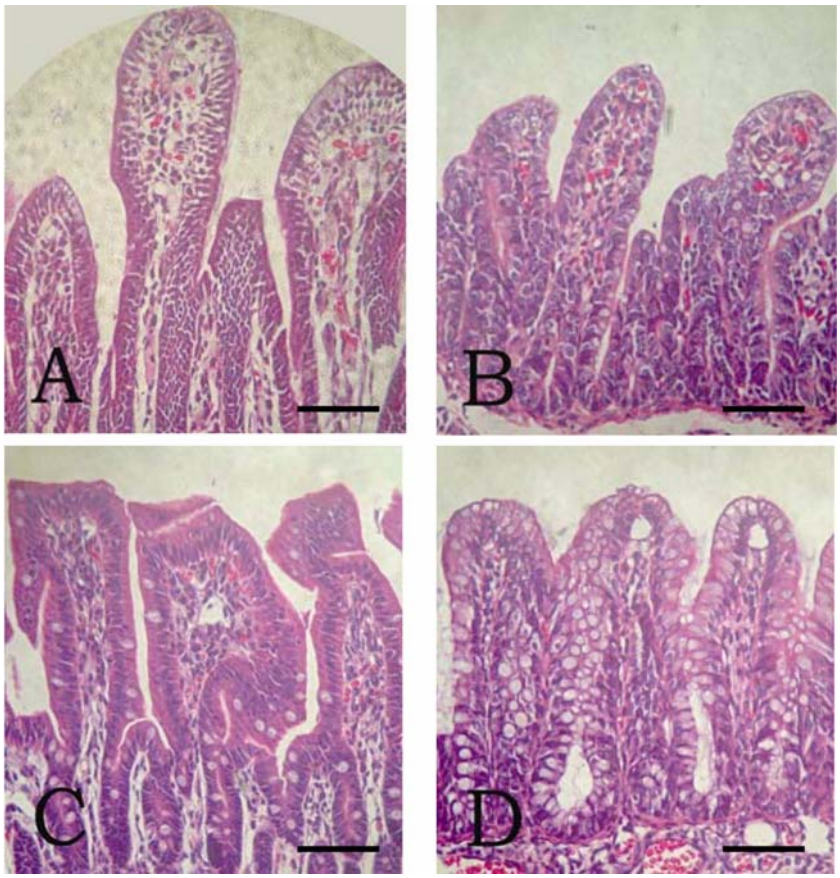

Fig. 6. Jejunum from lactobacillus inoculated piglets after challenging with virulent PEDV. Jejunum were collected from live (A) and dead (B) piglets orally inoculated for four days with *Lactobacillus reuteri* HY25101 and colostrums containing PEDV specific IgA before challenge and from live (C) and dead (D) piglets orally inoculated for four days with *Lactobacillus johnsonii* HY25103 and the colostrums before challenge. H&E stained. Bar = 100 μm.

Discussion

Probiotics including lactic acid bacteria are non-pathogenic microorganisms that exert beneficial roles on gastrointestinal diseases. It has been claimed that probiotics might help host by both improving gut microbial equilibrium and enhancing immunity [5, 16]. Indeed, *L. reuteri* HY25101 administration was obviously effective for prevention of PED in this study. In repeated independent *in vivo* experiments, *L. reuteri* HY25101 inoculated groups showed higher survival rate than those of other groups. However, survival rate of *L. reuteri* HY25101 administered groups were contradictory. Survival rate of *L. reuteri* HY25101 alone administered group I was 100% until 6 dpc in Exp 1, but survival rate of the group I coadministered with *L. reuteri* HY25101 and colostrums containing PEDV specific IgA was 45.5% until 6 dpc in Exp 2. When Exp 1 was performed, there might be some errors in virulent virus challenge procedure. It has been still unknown *L. reuteri*'s mode of preventive action on PEDV. Some studies have been characterized other strain of *L. reuteri*. *L. reuteri* 1063 strain, isolated from pig jejunal tissue, was a strongly autoaggregative strain having a relatively hydrophobic surface [17] and it was suggested that the autoaggregation might be a key factor to determine selection criteria for probiotics. *L. reuteri* 104R strain showed a high affinity for porcine small intestine mucus and gastric mucin. [10]. These results suggested that the beneficial effects of *L. reuteri* HY25101 might be derived by competition with pathogens for nutrients and adhesion sites. In addition

to this, *L. reuteri* HY25101 might also affect mucosal immune system in the gut. When probiotics made contact with intestinal epithelial cells or immune cells in Peyer's patches or lamina propria of intestine, the associated immune cells such as monocytes, macrophages and dendritic cells initiated innate immune response against these antigenic stimulations. After B cell activation, production of IgA antibody increased at mucosal surfaces [5, 11, 14]. In agreement with these reports, *Lactobacillus* GG enhanced nonspecific and rotavirus specific IgA secreting cell response [6] and *in vivo* studies in conventional animals revealed that *L. casei* CRL 431 strain induced innate immunity with an influence in the clonal expansion of IgA producing B cell population [3]. An important component of immunity at mucosal surfaces is the secretory IgA. Secretory IgA in milk helps to protect piglet from intestinal pathogens while it is in nursery stage. In Exp 2 the preventive effect of LAB on PEDV was investigated in suckling piglets possessing PEDV specific mucosal immunity. Intestinal PEDV specific IgA level of piglets was not investigated because detection of intestinal IgA level from piglets was very difficult. However administration of the recombinant yeast into pregnant sows induced PEDV specific IgG level in serum and IgA level in colostrums and these results supposed that the IgA in colostrums was transferred to piglets and this probably developed passive immunity against PED in the piglets. The jejunum from dead piglets possessing only passive immunity for PEDV of Exp 1 was more severely damaged and destructed than those of dead piglets of Exp 2. These results suggested that passive mucosal immunity by vaccination to pregnant sow might be not sufficient to completely protect piglet against PEDV infection and coadministration of lactobacilli with vaccine could generate more efficient protection than vaccine alone administration. Further studies were needed to confirm the improvement of cytokine production and population of antibody secreting cells in lymphoid tissue of small intestines after inoculation of *L. reuteri* HY25101. Colonization level of *L. reuteri* HY25101 to intestinal mucosal surface was also needed to investigate. In conclusion, results of this study suggested that dietary treatment using *L. reuteri* HY25101 could reduce diarrheal problem and mortality rates caused by PEDV in suckling pigs.

Acknowledgments

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