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Changhoon Park

Jiwoon Jeong

Kyuhyung Choi

Duck-Il Shin

A Rong Jeong

See next page for additional authors

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Short communication

In vivo Activity of *Lactobacillus plantarum* on Reduction in Enterotoxigenic *Escherichia coli* and Shiga Toxin-Producing *E. coli* in Postweaning Pigs

Changhoon Park[1] Jiwoon Jeong[1] Kyuhyung Choi[1] Duck-Il Shin[2] A Rong Jeong[2]

Sung-Hoon Kim[2] Chanhee Chae[*1]

Abstract

The objective of this study was to determine the effect of *Lactobacillus plantarum* on enterotoxigenic *Escherichia coli* (ETEC) and Shiga toxin-producing *E. coli* (STEC) in order to reduce lesions and fecal shedding for the control of colibacillosis and edema disease in postweaning pigs. ETEC K88 colonized in greater abundance on the surface of the small intestinal tract of non-*L. plantarum*-fed pigs compared to *L. plantarum*-fed pigs. Reduced intestinal adherence and fecal shedding of ETEC K88 was observed in the *L. plantarum*-fed pigs, whereas brain lesions and fecal shedding of STEC F18 remained unchanged. The results of this study demonstrated that *L. plantarum* appeared to have beneficial effects on the colibacillosis but not edema disease in postweaning pigs.

Keywords: enterotoxigenic *Escherichia coli*, *Lactobacillus plantarum*, Shiga toxin-producing *E. coli*

[1]*College of Veterinary Medicine, Seoul National University, 1Gwanak-ro, Gwanak-gu, Seoul 151-742 Republic of Korea*
[2]*College of Oriental Medicine, Kyunghee University, 1 Hoegi-dong, Dongdaemun-Gu, 130-701, Seoul, Republic of Korea*
[*]***Correspondence:*** *swine@snu.ac.kr*

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) and Shiga toxin-producing *E. coli* (STEC) are major causes of diarrhea and edema disease, respectively, in postweaning pigs. ETEC are known to contain at least 2 types of virulence factors: fimbriae and enterotoxins (heat-labile toxin; LT, and heat-stable toxin; ST). STEC also possess fimbriae and Shiga toxin (STx). Colonization (fimbriae) and toxin (enterotoxin and STx) production must occur together for disease (colibacillosis and edema disease) to ensue (Fairbrother and Gyles, 2012). K88 (F4), F18, and Stx are widely prevalent among *E. coli* isolated from postweaning pigs with colibacillosis and edema disease (Kwon et al., 2002).

Despite the fact that colibacillosis and edema disease can be controlled through good management practices and high hygiene standards (Fairbrother and Gyles, 2012), frequent immediate solution is to treat the animals with antibiotics. Antibiotic feed additives have been commonly used to treat colibacillosis and edema disease in pigs. However, the overuse of antibiotics as treatment and prophylactics purpose has contributed to the emergence and dissemination of antibiotic resistance genes (de Been et al., 2014). In an effort to reduce antibiotic use in animals, use of probiotics as non-antibiotic feed additives is an alternative in order to control colibacillosis and edema disease. Several *Lactobacillus* species have been reported to be effective in controlling colibacillosis (Nemcová et al., 2007; Brown, 2011; Suo et al., 2012; Guerra-Ordaz et al., 2014; Yang et al., 2014). However, the effect of *Lactobacillus* species against porcine STEC is unknown. Therefore, the objective of this study was to determine the effect of *L. plantarum* on ETEC and STEC in order to reduce lesions and fecal shedding to control colibacillosis and edema disease in postweaning pigs.

Materials and Methods

Lactobacillus plantarum strain (Genebiotech Co. Ltd., Seoul, Korea) isolated from a pickled chili was used to prepare the solid-state fermentation as follows: *L. plantarum* was cultured at 37°C in sterile MRS medium. Starter culture of *L. plantarum* was prepared by static culture with MRS broth at 37°C for 24 h. Fermentation was conducted in sterile stainless vessels (9×29 cm) containing 400 gram of substrate (roasted soybean flour) with a final moisture content of 45% and 2% starter culture at 37°C for 32 h. At the end of the experiments, all of the fermented substrates collected in one flask were ten-fold diluted in sterile phosphate-buffered saline (PBS; 0.144 % Na_2HPO_4, 0.024 % KH_2PO_4, pH 7.0), and the suspended viable cell concentration was determined. The final viable cell concentration was up to 3.0×10^9 CFU/gram.

The *E. coli* strain O8:K88$^+$LT$^+$ST$^+$ (ETEC K88) and *E. coli* strain O141:F18$^+$Stx$^+$ (STEC F18) used in this study were isolated from colibacillosis and edema disease outbreaks in postweaning pigs in Korea. The infection inoculum was cultured for 16 h at 37°C in BHI broth with slow agitation ($1 \times g$) in an orbital incubator to reach a final concentration of 3×10^9 CFU/ml.

Thirty pigs of 21 days of age were purchased from a commercial farm historically free of colibacillosis and edema disease outbreaks and from sows that had not been vaccinated against *E. coli*. All pigs were negative for rotavirus, coronavirus, ETEC according to routine polymerase chain reaction (PCR) testing from fecal samples. The thirty pigs were randomly allocated into 6 groups (n = 5 per group) using the random number generation function in Excel (Microsoft Corporation, Redmond, WA, USA). Pigs in groups 1, 2 and 3 received a commercial feed (Maxfeed Feeding Company, Bundang, Korea) supplemented with powder of *L. plantarum* (2% of total amount of feed). Pigs in group 4, 5, and 6 received only commercial feed. At 49 days of age (0 days post-inoculation; dpi), a single 3 ml oral dose (3×10^9 CFU/ml) of the *E. coli* O8:K88(F4)$^+$LT$^+$ST$^+$ strain was administered to each pig in groups 1 and 4 by oral gavage. A single 3 ml oral dose (3×10^9 CFU/ml) of the *E. coli* strain O141:F18$^+$STx$^+$ was administered to each pig in groups 2 and 5 by oral gavage. The pigs were monitored daily for physical symptoms. Fecal diarrhea scores were recorded by visual assessment of each subject using a 5-point scoring system (1–5), the extremes being hard (score 1) and watery fecal excrements (score 5) as previously described (Konstantinov et al., 2008). Morbidity and mortality rates were also recorded. Five pigs from each group were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 21 dpi as previously described (Beaver et al., 2001). Tissues were collected from each pig at necropsy. All of the methods were previously approved by the Seoul National University Institutional Animal Care and Use Committee.

Fecal samples were collected at 1, 2, 3, 7, and 14 dpi to evaluate whether the levels of ETEC K88 and STEC F18 in the feces were influenced by the *L. plantarum* supplement using coliform count and real-time PCR. Fecal coliform count was performed as previously described (Bosi et al., 2004). Real-time PCR was performed to quantify the ETEC K88, STEC F18, and *L. plantarum* genomic copy numbers as described elsewhere (Haarman and Knol, 2006; West et al., 2007). Prior to statistical analysis, all real-time PCR results were transformed to $\log_{10}$ values. Normality of the distribution of the examined variables was evaluated by the Shapiro-Wilk test. Continuous data (fecal coliform count, ETEC K88 and STEC F18) were analyzed with a repeated analysis of variance (ANOVA). If the repeated measures ANOVA showed a significant effect, Tukey's multiple comparison test was performed at each time point. Discrete data (fecal diarrhea score) were analyzed by Mann-Whitney test. A value of $p<0.05$ was considered significant.

Results and Discussion

Fecal diarrhea scores were monitored daily over 21 dpi. The non-*L. plantarum*-fed pigs challenged with ETEC K88 (group 4) exhibited moderate diarrhea (n = 3/5) at 4 dpi lasting for 4 days. In contrast, only the *L. plantarum*-fed pigs challenged with ETEC K88 (group 1) were observed to have mild diarrhea at 4 dpi. Fecal diarrhea scores were significantly ($p<0.05$) lower

in the *L. plantarum*-fed pigs challenged with ETEC K88 (group 1) than the non-*L. plantarum*-fed pigs challenged with ETEC K88 (group 4) at 7 dpi. Fecal diarrhea scores were significantly (p<0.05) lower in the *L. plantarum*-fed pigs (group 3) and the non-*L. plantarum*-fed pigs (group 6) than the non-*L. plantarum*-fed pigs challenged with ETEC K88 (group 4) from 4 to 7 dpi. There was no diarrhea in the *L. plantarum*-fed pigs challenged with STEC F18 (group 2) and the non-*L. plantarum*-fed pigs challenged with STEC F18 (group 5).

There were no significant differences in the fecal coliform counts among the 6 groups throughout the experiment. The effect of *L. plantarum* powder supplementation on ETEC K88 levels in the fecal samples was also examined. ETEC K88 was reduced significantly (p<0.05) in the fecal samples of the *L. plantarum*-fed pigs challenged with ETEC K88 (group 1) compared to the samples of the non-*L. plantarum*-fed pigs challenged with ETEC K88 (group 4) at 3 and 7 dpi (Fig 1). There was no significant difference in the number of genomic copies of STEC F18 between the *L. plantarum*-fed pigs challenged with STEC F18 (group 2) and the non-*L. plantarum*-fed pigs challenged with STEC F18 (group 5) (Fig 2). ETEC K88 and STEC F18 were not detected in the *L. plantarum*-fed pigs (group 3) and the non-*L. plantarum*-fed pigs (group 6) throughout the experiment.

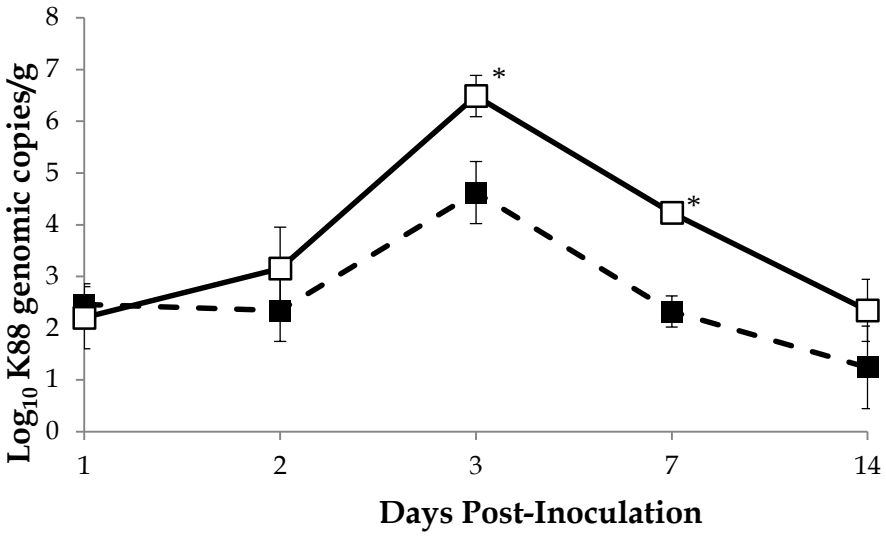

Figure 1 Mean values of the genomic copy number of enterotoxigenic *Escherichia coli* (ETEC) in fecal samples at various days post-inoculation (dpi). *Lactobacillus plantarum*-fed pigs challenged with ETEC K88 (group 1, ■) and non-*L. plantarum*-fed pigs challenged with ETEC K88 (group 5, □). *Significant (p<0.05) difference between groups.

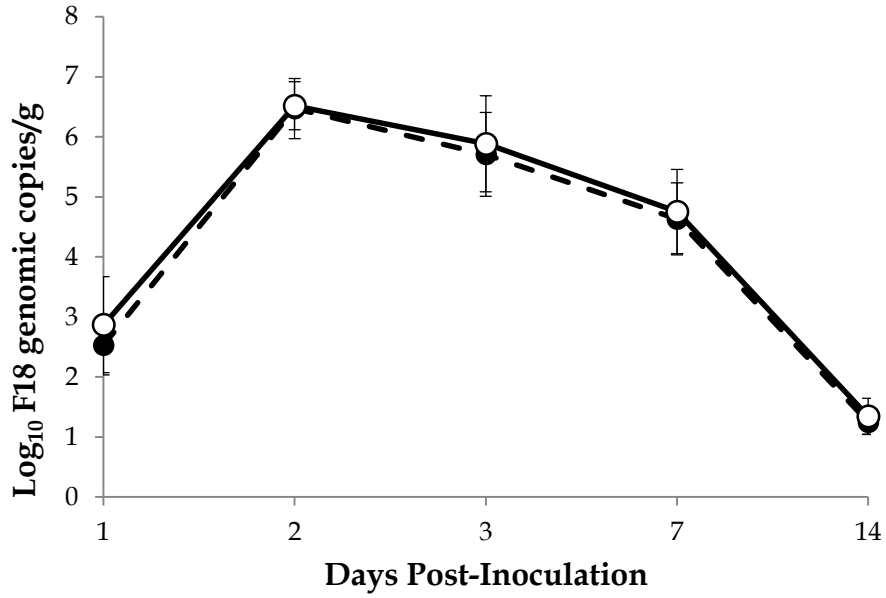

Figure 2 Mean values of the genomic copy number of Shiga toxin-producing *E. coli* (STEC) in fecal samples at various days post-inoculation (dpi). *Lactobacillus plantarum*-fed pigs challenged with STEC F18 (group 2, ●) and non-*L. plantarum*-fed pigs challenged with STEC F18 (group 5, ○). *Significant (p<0.05) difference between groups.

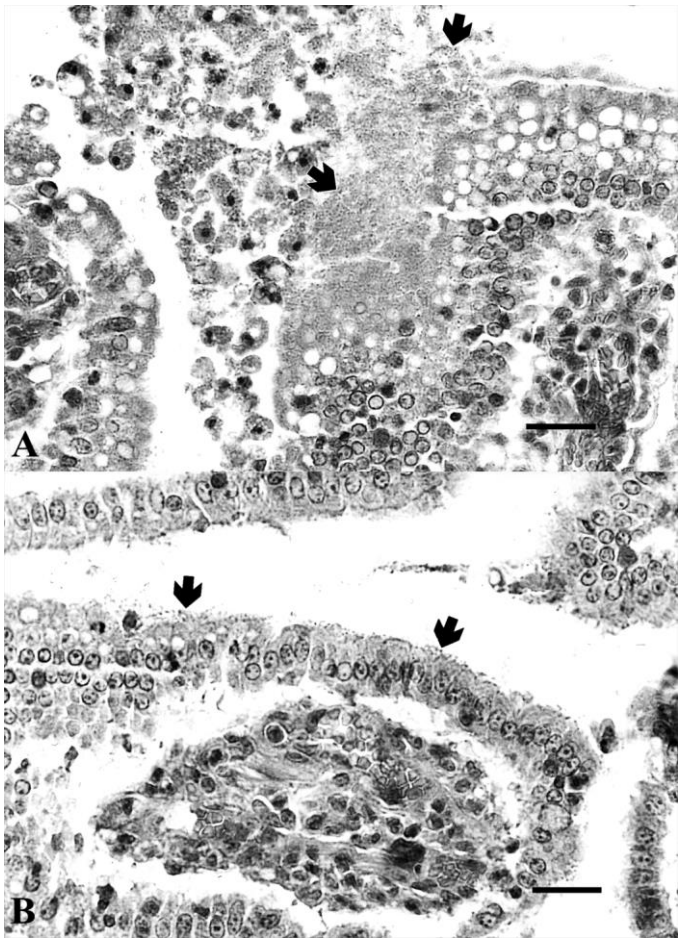

Figure 3 Enterotoxigenic *Escherichia coli* (ETEC) K88 colonized in greater abundance to surface of ileal intestinal tract of non-*L. plantarum*-fed pigs challenged with ETEC K88 (arrows) (A) than *L. plantarum*-fed pigs challenged with ETEC K88 (arrows) (B) at 14 days post-inoculation. Hematoxylin-eosin stain. Bar = 100 μm.

Histopathologically, ETEC K88 closely adhered to enterocytes on the mucosal surface. ETEC K88 colonized in greater abundance on the surface of jejunal and ileal intestinal tract of the non-*L. plantarum*-fed pigs challenged with ETEC K88 (Fig 3A) than the *L. plantarum*-fed pigs challenged with ETEC K88 (Fig 3B). Microscopic hemorrhages were seen in the brain of the *L. plantarum*-fed pigs challenged with STEC F18 and the non-*L. plantarum*-fed pigs challenged with STEC F18 at various sites, including subcortical frontal cerebrum, cerebellar folia, cerebellar peduncles, and brain stem. Hemorrhagic foci were often in association with microinfarcts in capillaries and venules.

L. plantarum was never detected in the fecal samples from the non-*L. plantarum*-fed pigs (groups 4, 5, and 6) throughout the experiment while it was present in the samples from the *L. plantarum*-fed pigs (groups 1, 2, and 3) by real-time PCR (data not shown).

The beneficial effect of probiotic bacteria has also been associated with their metabolites, which stimulate the colonization and reproduction of *Lactobacilli* and other anaerobic bacteria in the mucosa, and suppress the enterobacterial numbers in the intestines (Chang et al., 2001; Huang et al., 2004). The reduced intestinal adherence and fecal shedding of ETEC K88 observed in the *L. plantarum*-fed pigs may suggest colonization of the gut mucosa by the probiotic bacteria, thereby inhibiting the adhesion of ETEC K88 to the intestinal mucosa. This finding supports previous studies which observed that deleterious effects caused by ETEC K88 were inhibited in *L. plantarum* treated pigs (Lee et al., 2012; Yang et al., 2014). The inhibition of bacterial adherence via the receptor of intestinal mucosa is a promising method to prevent ETEC infection (Shoaf-Sweeney and Hutkins, 2008).

Brain lesions and fecal shedding of STEC was not significantly reduced after the treated pigs were challenged with STEC F18 in the present study. Our results are in contrast with a previous finding in which reduction in fecal shedding of non-O157 STEC was observed in sheep that received probiotic mixture which contained several Lactobacillus species, *Streptococcus thermophilu*s and *Enterococcus faecium* (Rigobelo et al., 2014). These results suggest that different *Lactobacillus* species have different effect against ETEC strains (Zhang et al., 2010; Yang et al., 2014).

Selection of a proper animal is critical when evaluating the effect of *L. plantarum*. The pigs used in this study were phenotypically classified as susceptible to ETEC K88 and STEC F18 adhesion to surface of intestinal tract by an *in vitro* test as previously described (Baker et al., 1997). Therefore, the effectiveness of the treatment was due to the interaction of *L. plantarum* with the sensitivity of intestinal tract to ETEC K88 and STEC F18 adhesion. The results of this study demonstrate that *L. plantarum*

appears to have a beneficial effect on colibacillosis but not edema disease in postweaning pigs. Since the overuse of antimicrobials has resulted in increase in antimicrobial resistance and failure in the treatment of edema disease in Japanese farms (Uemura et al., 2003), future work is needed to identify other *Lactobacillus* species that could have a beneficial effect on edema disease caused by STEC F18.

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บทคัดย่อ

การศึกษาประสิทธิภาพการทำงานภายในร่างกายของ *Lactobacillus plantarum* ต่อ Enterotoxigenic และ Shiga-toxin producing *Escherichia coli* ในลูกหมูหย่านม

*Changhoon Park[1] Jiwoon Jeong[1] Kyuhyung Choi[1] Duck-Il Shin[2] A Rong Jeong[2] Sung-Hoon Kim[2] Chanhee Chae[*1]*

การศึกษานี้มีวัตถุประสงค์เพื่อประเมินผลของ *Lactobacillus plantarum* ต่อ enterotoxigenic *Escherichia coli* (ETEC) และ Shiga toxin-producing *E. coli* (STEC) ในการช่วยลดรอยโรคและการปลดปล่อยเชื้อทางอุจจาระ เพื่อช่วยในการควบคุมโรค colibacillosis และภาวะบวมน้ำในลูกสุกรหลังหย่านม การศึกษาครั้งนี้พบว่า ETEC K88 ก่อตัวเป็นกลุ่มบนผิวของลำไส้เล็กสุกรกลุ่มควบคุมมากกว่ากลุ่มสุกรที่ได้รับ *L. plantarum* นอกจากนี้ยังพบว่าสามารถลดการเกาะตัวและการขับออกของเชื้อ ETEC K88 ทางอุจจาระในกลุ่มสุกรที่ได้รับ *L. plantarum* แต่ในทางตรงกันข้ามไม่พบการเปลี่ยนแปลงของ STEC F18 ในการก่อให้เกิดรอยโรคที่สมองและการขับออกของเชื้อทางอุจจาระ การศึกษาครั้งนี้แสดงให้เห็นว่า *L. plantarum* มีแนวโน้มช่วยลดโอกาสเกิด colibacillosis แต่ไม่ช่วยในกรณีของภาวะบวมน้ำในลูกสุกรหลังหย่านม

คำสำคัญ: enterotoxigenic *Escherichia coli Lactobacillus plantarum* Shiga toxin-producing *E. coli*

[1]*College of Veterinary Medicine, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151-742 Republic of Korea*
[2]*College of Oriental Medicine, Kyunghee University, 1 Hoegi-dong, Dongdaemun-Gu, 130-701, Seoul, Republic of Korea*
***ผู้รับผิดชอบบทความ E-mail:** swine@snu.ac.kr*