離乳期子豚を用いた腸管毒血症大腸菌実験感染系におけるEnterococcus faecalis殺菌菌体(EC-12)の低濃度における浮腫病予防性評価

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FULL PAPER *Anatomy*

Evaluation of the Low Dose Level of a Heat-Killed and Dried Cell Preparation of *Enterococcus faecalis* to Prevent Porcine Edema Disease Using Experimental Infection Model with Enterotoxcemic *Escherichia coli* in Weaning Pigs

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ABSTRACT. Porcine edema disease (ED) is caused by Shiga toxin 2e-producing *Escherichia coli* (STEC). ED has become frequent in pig farms, and the use of antimicrobials has resulted in the development of antimicrobial-resistant STEC. Accordingly, the use of materials other than antimicrobials is requested for the prevention of ED. Oral administration of a heat-killed and dried cell preparation of *Enterococcus faecalis* strain EC-12 (EC-12) to weaning pigs was previously demonstrated to decrease animal mortality in a STEC-contaminated farm at 0.05% (w/w) dose level. In this study, pigs experimentally infected with STEC were used as a model for ED to evaluate the low dose level of EC-12 to prevent ED. Fifteen 21-day-old pigs were divided into 5 groups: STEC challenge with the basal diet, STEC challenge with EC-12 supplemented at 0.005, 0.01, or 0.05% (w/w) to the basal diet, and no STEC challenge with the basal diet. The challenge was carried out when the animals were 25, 26, and 27 days old using STEC contained in capsules resistant against gastric digestion. All pigs were euthanized at 32 days of age. The daily weight gain, feed conversion ratio, and palpebral edema were improved by supplementation with 0.05% EC-12, but not by the low dose levels. Accordingly, 0.05% level of supplementation was needed for EC-12 to improve clinical symptoms in weaning piglets infected by STEC.

KEY WORDS: *Enterococcus faecalis* strain EC-12, enterotoxcemic *Escherichia coli*, experimental infection, porcine edema disease.

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Edema disease (ED), a sporadic disease of weaning pigs, is caused by host-adapted *Escherichia coli* producing Shiga toxin 2e (Stx 2e) [2]. Stx 2e-producing *E. coli* (STEC) colonizes the lower small intestine in pigs by F18 fimbriae and develops ED by locally produced Stx 2e [2, 3]. ED has recently become frequent in Japan [24, 26], but its control remains difficult. Antimicrobial therapy is a major curative treatment for pigs infected with ED. However, frequent antimicrobial treatment has induced antimicrobial resistance to STEC in Japanese farms [5, 21]. Furthermore, the use of some antimicrobials, such as ampicillin and fosfomycin, possibly aggravates the ED symptoms [22]. Accordingly, the application of so-called non-antimicrobials, such as probiotics and prebiotics, is requested for the prevention or treatment of ED, most particularly, in farms where antimicrobial-resistant STEC has been identified.

In our previous study, dietary a heat-killed and dried cell preparation of *Enterococcus faecalis* strain EC-12 (EC-12) administration at 0.05% level to weaning pigs significantly reduced the motility in a farm severely contaminated with enterotoxigenic *E. coli* (ETEC) and STEC [18, 20]. From the economical point of view, a lower dose level of EC-12 is requested by farmers to obtain the better animal performance with smaller dispences for hygienic control. Accordingly, in this study, we used an experimental STEC infection model [19] to evaluate the lower dose-levels of EC-12 than 0.05% to prevent ED.

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MATERIALS AND METHODS

Animals and basal diets: Fifteen (ten female and five male) 21-day-old crossbred (Landrace × Large white × Duroc) pigs weighing approximately 4.7 kg were purchased from a commercial pig farm. The animals were healthy and did not receive any antimicrobials prior to the study. They were checked to be negative for infection with STEC by the method as described previously [19]. The pigs were housed in pens at the KYODOKEN Institute, Kyoto, Japan. They were divided into five groups of one male and two females to obtain a similar mean body weight, and each group was housed in a pen. The temperature was maintained with brooders at 30°C (determined on the floor just under the brooder).

The animals were fed with a commercial experimental diet for weaning pigs (SDS No.1; Nippon Formula Feed, Yokohama, Japan). This basal diet was free from intestinal microbiota modifiers, such as antimicrobials and pre- and pro-biotics. The nutrient composition of this diet was the same as described previously [17]. The diet and water were given *ad libitum* throughout the study. The experimental animals were handled in accordance with the guidelines for

animal studies of the KYODOKEN Institute.

EC-12, a commercial product of a heat-killed and dried cell preparation of *E. faecalis* strain EC-12 (Combi Corporation, Saitama, Japan), was used. EC-12 was incorporated at 0.005, 0.01, or 0.05 (% w/w) into the diet.

Source and nature of STEC: *E. coli* strain 1362-1 sero group O139, obtained from Dr. M. Sueyoshi, Miyazaki University, Miyazaki, Japan, was used to induce ED in pigs in the same manner as described previously [19]. Strain 1362-1 is hemolytic and the producer of Shiga toxin 2e, a heat-stable enterotoxin, and the F18 fimbriae, a mediator of colonization on the intestine of weaning pigs [3].

Induction of ED and sampling protocols: Five experimental groups were as follows: STEC infection with the basal diet (CI group), STEC infection with a basal diet supplemented with EC-12 at 0.005% (w/w) (E-0.005 group), STEC infection with a basal diet supplemented with EC-12 at 0.01% (w/w) (E-0.01 group), STEC infection with a basal diet supplemented with EC-12 at 0.05% (w/w) (E-0.05 group), and no infection with STEC with the basal diet (CNI group). Each group was fed the respective diet throughout this study. After four days of introduction, oral administration of capsules containing 4.0×10^8 CFU of STEC was conducted for all groups except the CNI group (one capsule per animal daily) [19]. Capsules containing saline were orally administered to the pigs in the CNI group. The capsules were given to the pigs at 10 a.m. for three consecutive days (d0, d1, and d2). The clinical signs of the disease, such as palpebral edema, neurologic impairment, lateral recumbency, loss of appetite and vitality, respiratory problems, and diarrheal feces, were checked daily. The body weight was measured on d0 and d7. Remaining feed was weighed on d7. On d7, all pigs were euthanized by exsanguination under general anesthesia with intramuscular injections of ketamine hydrochloride (Veterinary Ketalar 50, Sankyo, Tokyo, Japan) and azaperone (Stresnil, Sankyo, Tokyo, Japan). After a midline incision, the entire intestine was removed and separated into the jejunum, the ileum, the cecum, and the centripetal turns of spinal loop of colon subsequently to string ligation. Each segment of the intestine was longitudinally incised, and the luminal contents were collected from the ileum and the cecum. Mid-portions of the jejunum, the cecum, and the centripetal turns of spinal loop of colon and the mid- and end-portions of the ileum were fixed in a 10% (v/v) phosphate-buffered formalin solution. The lung, kidney, liver, spleen, and mesenteric lymph node (MLN) were also collected and fixed in phosphate-buffered formalin.

Bacteriologic studies: The real-time polymerase chain reaction (PCR) on *stx2e* was adopted to detect the challenged STEC in ileal and cecal digesta with the Rotor-Gene system 6200 (Nippn TechnoCluster, Tokyo, Japan). For the template DNA, bacterial genomic DNA was extracted from the digesta using the DNA isolation kit for feces (ISOFECAL, Nippon Gene, Tokyo, Japan). Real-time PCR was performed with a SYBR *Premix Ex Taq*™ (Takara Bio Inc., Shiga, Japan) and primers JMS2F and JMS2R [4]. Each 20 μL of the PCR mixture contained 10 μL of SYBR Premix Ex Taq™, 0.2 μM of each primer, and 2 μL of extracted bacterial DNA. The samples were amplified using the following thermal program: initial denaturation at 95°C for 30 s and 40 thermal cycles of 95°C for 4 s, 55°C for 10 s, and 72°C for 10 s. The fluorescent signal from the samples was acquired at the end of the extension step. The detection level of *stx2e* was calibrated with the known number (10^2 to 10^7 cells/mL) of STEC.

Temperature Gradient Gel Electrophoresis (TGGE) analysis: The diversity of the intestinal ileal and cecal microbiota was further examined in this study using TGGE analysis. PCR on the bacterial 16S rRNA gene was performed as described by Inoue *et al.* [6] using primers U968-GC and L1401 [12]. The D-code system (Bio-Rad, Tokyo, Japan) was used for the sequence-specific separation of these PCR products. Electrophoreses and gel-image analyses were performed as described by Inoue *et al.* [6].

Organic acid concentration and pH analysis: Organic acid [short-chain fatty acid (SCFA), succinate, lactate, and formate] in ileal and cecal digesta was analyzed by ion-exclusion HPLC as described elsewhere [23]. The pH of the digesta was measured with a compact pH meter (twin B-211, Horiba, Kyoto, Japan).

Histological studies: Histological studies were similar to those described elsewhere [16, 19]. Briefly, the formalin-fixed intestinal tissues were further cut into cross sections of an approximate length of 10 mm. Each intestinal segment was embedded in paraffin wax. Micro sections that were 3-μm thick were prepared and stained with hematoxylin and eosin (HE) or Luna for light microscopic examination. Micro sections of the lung, kidney, liver, spleen, and MLN were also examined for microscopic analyses after HE staining.

The numbers of eosinophils were counted for the whole area of the mucosal tissue of the jejunum and the ileum at 200 × magnification in the Luna stain. The entire area (inch × inch) of the mucosa was measured using the NIH Image software [8] with a Macintosh personal computer (Apple Japan, Inc., Tokyo, Japan). Pictures for the image analyses were taken with a digital camera (DXM1200, Nikon, Tokyo, Japan) attached to a light microscope (ECLIPSE E1000M, Nikon).

Statistical analyses: Values are given as the means with standard deviation (n=3). The randomized blocked design 1-way ANOVA was used to analyze the differences in body weight gain, eosinophil numbers in the mucosa, and STEC numbers, the organic acid concentration and pH of the digesta. Tukey-Kramer's post-hoc comparison was used for multiple comparisons when needed. The Kruskal-Wallis test was used to analyze the differences of the clinical scores. Non-parametric Tukey-Kramer's post-hoc comparison was used for multiple comparisons when needed. The difference among means was considered significant at $p<0.05$ or showed a tendency to be significant at $p<0.1$ levels in all statistical analyses. All data were analyzed by StatLight 2000 (Yukms Co., Ltd., Tokyo, Japan) or Statcel [25].

RESULTS

Clinical score, body weight gain, and feed intake (Table 1): The clinical sign of palpebral edema was observed in the CI group from d3 to d7. The total clinical score of palpebral edema from d0 to d7 tended to be lower in the E-0.005, E-0.05, and CNI groups than in the CI group ($p<0.1$). Reduction in appetite was also observed in the CI group from d1 to d7. The total clinical score of appetite tended to be lower in the E-0.05 and CNI groups than in the CI group ($p<0.1$). Respiratory problems were only observed in the CI (from d5 to d7) and E-0.005 (from d4 to d7) groups. Diarrhea or loose feces were observed in the CI and E-0.01 groups from d1 to d7. Diarrheal feces were observed in one pig in the E-0.01 group on d2. Loss of vitality, neurologic impairment, and lateral recumbency were not observed in any pigs

Table 1. Effect of dietary EC-12 on clinical scores of the STEC-infected piglets[a)]

Scores[d)]	Group[e)]	Days after infection[b)]								Total score[c)]
		0	1	2	3	4	5	6	7	(d1–7)
Vitality[f)]	CI	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.3
	E-0.005	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.3
	E-0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	E-0.05	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	CNI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Appetite[g)]	CI	0.0	0.3	1.0	0.7	1.0	0.7	0.3	0.7	4.7^{a}
	E-0.005	0.0	0.0	0.3	0.7	0.7	0.7	0.3	0.3	3.0^{ab}
	E-0.01	0.0	0.0	0.3	0.3	0.3	0.3	0.0	0.3	1.7^{ab}
	E-0.05	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0^{b}
	CNI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0^{b}
Respiration[h)]	CI	0.0	0.0	0.0	0.0	0.0	0.7	0.3	0.7	1.7
	E-0.005	0.0	0.0	0.0	0.0	0.3	0.3	0.3	0.3	1.3
	E-0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	E-0.05	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	CNI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Feces[i)]	CI	0.0	0.3	0.3	1.0	1.0	0.7	0.3	0.7	4.3
	E-0.005	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	E-0.01	0.0	0.7	0.3	0.7	0.7	0.7	0.7	0.7	4.3
	E-0.05	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.7
	CNI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Palpebral edema[j)]	CI	0.0	0.0	0.0	0.7	0.3	0.7	0.3	0.3	2.3^{a}
	E-0.005	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0^{b}
	E-0.01	0.0	0.0	0.0	0.0	0.3	0.3	0.3	0.0	1.0^{ab}
	E-0.05	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0^{b}
	CNI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0^{b}
Neurologic impairment[k)]	CI	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.3
	E-0.005	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	E-0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	E-0.05	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	CNI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

a) Values are means, n=3.
b) STEC was infected at d0, d1 and d2.
c) Non-parametric Tukey-Kramer's post-hoc comparison was used for multiple comparisons when the Kruskal-Wallis test was significantly different. Means in a column without common letters tend to differ ($p<0.1$).
d) Lateral recumbency was not observed in all animals throughout this experiment.
e) CI, control diet with STEC infection ; E-0.005, EC-12 was incorporated in diet at 0.005%(w/w) and pigs were infected with STEC; E-0.01, EC-12 was incorporated in diet at 0.01% (w/w) and pigs were infected with STEC; E-0.05, EC-12 was incorporated in diet at 0.05% (w/w) and pigs were infected with STEC; CNI, control diet without STEC infection.
f) Vitality score: 0, good; 1, loose; 2, bad.
g) Appetite score: 0, good; 1, slightly poor; 2, poor.
h) Respiratory score: 0, normal; 1, slightly quick; 2, quick.
i) Fecal score: 0, normal; 1, loose stool; 2, moderate diarrhea; 3, severe diarrhea.
j) Edemal score in palpebra: 0, normal; 1, mild; 2, moderate; 3, severe.
k) Neurologic impairment score: 0, not impairment; 1, slightly; 2, moderate; 3, severe.

Table 2. Effect of dietary EC-12 on body weight gain, feed intake and feed conversion ratio of the STEC infected piglets[a)]

Group[b)]	Body weight gain[c)] (g/d)	Feed intake (g/d)	Feed conversion ratio
CI	-4.8 ± 83.7^{b}	138.6	–
E-0.005	42.9 ± 124.5^{ab}	121.0	2.82
E-0.01	57.1 ± 28.6^{ab}	147.1	2.58
E-0.05	204.8 ± 59.5^{a}	229.5	1.12
CNI	219.0 ± 70.5^{a}	263.3	1.20

a) STEC was infected at d0, d1 and d2.
b) See, Table 1.
c) Values are means ± SD, n=3. Tukey-Kramer's post-hoc comparison was used for multiple comparisons when 1-way ANOVA was significantly different. Means in a column without common letters differ significantly ($p<0.05$).

Table 3. Effect of dietary EC-12 on ileal and cecal organic acid concentrations in the STEC infected piglets[a)]

Intestine	Group[b)]	Organic acid concentration (mmol/kg digesta) Lactate	Acetate	Propionate	n-Butyrate	n-Valerate	pH
Ileum	CI	6.0 ± 5.4	1.6 ± 0.4	0.5 ± 0.5^{b}	0.4 ± 0.4	0.0 ± 0.0	7.1 ± 0.3
	E-0.005	39.3 ± 4.5	2.7 ± 1.4	0.6 ± 1.1^{b}	0.1 ± 0.2	0.4 ± 0.7	6.7 ± 0.3
	E-0.01	24.6 ± 16.1	2.4 ± 1.9	0.4 ± 0.4^{b}	0.5 ± 0.5	0.2 ± 0.4	6.8 ± 0.3
	E-0.05	20.4 ± 10.3	3.3 ± 0.5	1.3 ± 0.7^{ab}	0.2 ± 0.4	0.0 ± 0.0	6.9 ± 0.3
	CNI	45.0 ± 38.2	4.4 ± 2.9	2.6 ± 2.2^{a}	0.0 ± 0.0	0.0 ± 0.0	6.5 ± 0.4
Cecum	CI	12.4 ± 20.9	48.8 ± 29.7^{AB}	19.2 ± 11.9^{B}	10.0 ± 9.2	2.6 ± 1.8	6.5 ± 1.1
	E-0.005	1.5 ± 0.5	76.1 ± 11.5^{A}	42.7 ± 5.7^{A}	14.1 ± 11.8	3.3 ± 2.9	6.1 ± 0.7
	E-0.01	14.7 ± 12.2	35.4 ± 19.9^{B}	16.9 ± 11.0^{B}	7.4 ± 5.8	1.9 ± 2.1	6.5 ± 0.7
	E-0.05	9.8 ± 13.2	59.3 ± 6.4^{AB}	31.2 ± 4.8^{AB}	32.0 ± 21.3	6.9 ± 8.0	5.8 ± 0.4
	CNI	0.6 ± 0.5	67.1 ± 11.5^{AB}	38.7 ± 8.0^{A}	21.4 ± 4.8	7.3 ± 1.9	5.8 ± 0.2

a) Values are means ± SD, n=3. The randomized blocked design 1-way ANOVA was used to analyze the differences for each portion. Tukey-Kramer's post-hoc comparison was used for multiple comparisons when 1-way ANOVA was significantly different. Means for cecal acetate or cecal propionate without common capital letters differ significantly ($p<0.05$). Means for ileal propionate without common small letters tend to differ ($p<0.1$). Succinate, formate, iso-butyrate and iso-valerate concentrations were always trace to omit the consideration.
b) See Table 1.

throughout the study.

Body weight gain, feed intake, and feed conversion ratio (Table 2): Body weight decreased in the CI group during the experiment, whereas it increased in the other groups. The daily body weight gain was significantly larger in the E-0.05 and CNI groups than in the CI group ($p<0.05$). Feed intake (g/d/head) in the E-0.05 and CNI groups was twice as large as that in the CI, E-0.005, and E-0.01 groups. The feed conversion ratio was not calculated for the CI group, because their body weight did not increase. The feed conversion ratios were 2 times lower or more in the E-0.05 and CNI groups than in the E-0.005 and E-0.01 groups.

Detection of STEC: The *stx2e* gene was detected in the ileal and cecal digesta of all pigs that received STEC orally. The mean detection level of STEC in the cecal digesta ranged (cells/g) 9.0×10^5, 1.7×10^4, 1.6×10^6, and 2.2×10^6 in CI, E-0.005, E-0.01, and E-0.05 group, respectively. These values were not significantly different from each other. The mean detection level of STEC in the ileal digesta ranged 9.8×10^2, 5.2×10^2, 3.0×10^7, and 4.1×10^4 in CI, E-0.005, E-0.01, and E-0.05 group, respectively. There was no statistical significance.

Organic acid concentration and pH in the intestinal digesta (Table 3): The lactate concentration was not significantly affected by the dietary EC-12 at any portion of the intestine. On the other hand, the cecal acetate was significantly larger in the E-0.005 group than that in the E-0.01 and E-0.05 groups ($p<0.05$). Cecal propionate was significantly larger in the E-0.005 and CNI groups than in the CI and E-0.01 groups ($p<0.05$). However, the concentrations of these SCFA were not different among the experimental groups in the ileum. n-Butyrate and n-valerate were detected at only trace amounts (less than 0.5 mmol/kg digesta) in the ileum. The concentrations of these SCFA in the cecum were not different among the experimental groups. Other organic acids, such as succinate, formate, iso-butyrate, and iso-valerate, were detected in amounts of less than 2 mmol/kg digesta, therefore we omitted from considerations.

The pH of ileal digesta tended to be lower in the CNI group than in the CI group ($p<0.1$).

Histological evaluations: The level of mucosal erosion, ulcer, atrophy, hypertrophy, and villous loss in the small and

large intestines appeared normal in all pigs. However, swollen lymphoid follicles were observed in the small and large intestines in all pigs. The lymphoid follicles were particularly swollen in the end segment of the ileum. However, the swollen lymphoid follicles were not different among the experimental groups at any portion of the intestine. Edematous lamina propria was observed in the small and large intestines of all pigs. However, the level of edema was not different among the experimental groups at any segment of the intestine. The lung appeared abnormal in some pigs in this study, but their abnormalities were not different among the groups. Other organs, such as the kidney, liver, and spleen, and tissue (MLN) appeared normal in all pigs.

Dendrogram from TGGE analysis (Fig. 1): The diversity of the luminal microbiota in the ileum and cecum was evaluated by a dendrogram created from the PCR-TGGE band profiles (Fig. 1). The dendrogram of the ileal digesta was composed of three clusters (Fig. 1A). A cluster contained the profiles of three pigs of the CI group and one from the CNI group. The other cluster contained profiles of 2 pigs from the E-0.005 group, two from the E-0.01 group, and one from E-0.05 group. The remaining cluster contained the profiles of 2 from the E-0.05 group, two from the CNI group, one from E-0.005 group, and one from the E-0.01 group. On the other hand, the profiles of the cecal microbiota were less clearly separated; for example, the profiles of CI pigs were combined with those of other groups of pigs (Fig. 1B).

Numbers of eosinophil in the small intestine: The mean number (cells/inch^2) of eosinophil in the jejunal mucosa was 263, 998, 435, 146 and 302 in CI, E-0.005, E-0.01, E-0.05, and CNI group respectively. These values were not significantly different from each other. The mean number of eosinophil in the ileal mucosa was 750, 1,082, 690, 709 and 417 in CI, E-0.005, E-0.01, E-0.05 and CNI group, respectively. There was no statistical significance.

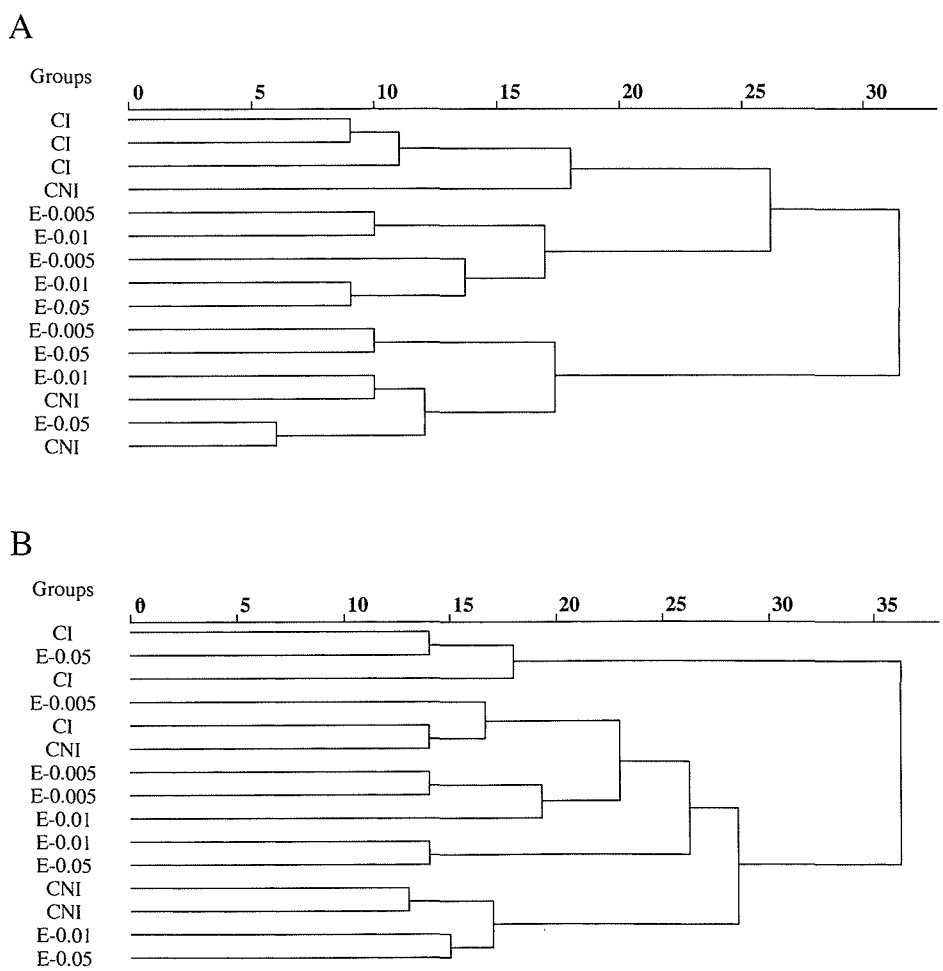

Fig. 1. Dendrogram based on the TGGE profiles of bacterial 16S rDNA in the ileal (A) and cecal (B) digesta of pigs. The cluster analysis was conducted using hierarchical clustering with Euclidean square distances by an Excel macro program.

DISCUSSION

The present technique based on a capsule resistant to gastric digestion was successful to promote experimental STEC infection, as previously reported [19]. This procedure was demonstrated to be useful to evaluate the preventive effect of materials against porcine ED. In the present experiment, relatively low STEC challenge was adopted. Therefore, histological observation on the intestinal tissue showed no clear difference between the group as shown in our previous study [19]. Accordingly, effect of STEC infection was evaluated by the clinical scores and body weight gain. A higher dose [0.05% (w/w)] of dietary EC-12 reduced the level of clinical symptoms of ED as low as those in the healthy control pigs (CNI group). As a result of such improvement, the body weight gain and feed conversion ratio were maintained as those recorded in the healthy control pigs (CNI group). On the other hand, the clinical symptoms of ED and body weight gain were not improved by lower dose levels [0.005% and 0.01% (w/w)] of EC-12.

STEC was detected in the ileum and the cecum of all experimentally infected pigs; luminal numbers of STEC were not affected by EC-12 administration. This means that dietary EC-12 itself does not affect the viability of STEC. Therefore, other, most likely indirect, mechanisms must be involved in the improvement of the growth performance of STEC-infected pigs observed in the present study.

Lactic acid bacteria (LAB) including EC-12 prevent pathogenic diarrhea such as colitis and rotavirus infection in the weaning pigs [7, 13, 18]. As shown by Fig. 1, EC-12 affected the luminal microbiota in the small intestine. An increase in the lactate concentration in the ileal digesta by dietary EC-12 administration suggested that indigenous LAB might increase in the small intestine. Since EC-12 is known to stimulate indigenous LAB, such as bifidobacteria in the human intestine [14], this hypothesis is reasonable. Shu *et al.* [13] suggested that immunostimulation by LAB is effective to improve the resistance to diarrheal disease in the weaning pigs. EC-12 interacts with host immune systems to stimulate the production of proinflammatory cytokines of the murine macrophage [9]. The stimulation of $CD8^+$ T-cells has also been observed [9]. These findings suggest that dietary EC-12 together with indigenous LAB which were stimulated by dietary EC-12 can stimulate an inflammatory reaction in the small intestine of pigs. Therefore, a possible mechanism of dietary EC-12 to prevent ED in pigs involves the modulation of the immunologic function in the intestine.

Physiological responses of intestinal tissues, immunological responses in particular, to LAB may show a dose-dependency. However, the response must not be a proportional to the dose level. The results obtained from the *in vivo* experiment in which production of colony stimulating factor (CSF) was determined with different dose-level of LAB showed a sigmoidal-type response [10]. Sigmoidal curve with a narrow dose range for log-phase increase in CSF suggests the optimal dose-level of LAB may not be broad. Higher dose may give a rapid saturation of the response and lower dose cannot lead the response. Lower dose levels of EC-12 in the present study may not be strong enough to promote immune reponses to STEC. In our preliminary field research, 0.025% dietary EC-12 administration had no effect to prevent ED in piglets, while 0.05% EC-12 reduced mortality of piglets by ED. Interestingly, 0.1% EC-12 showed no further improvement [20].

Other possible mechanism is an inhibition of attachement of ETEC to the mucosa [7]. These authors reported that the adhesion of ETEC to the small intestine mucus was affected by the presence of *E. faecium*. The adhesion of ETEC to the intestinal mucus was nearly completely inhibited by the presence of over 10^9 cells/mL of *E. faecium*. However, this inhibition became ineffective as the level of *E. fecium* decreased and completely lost at the level of 10^7 cells/mL of *E. faecium*. In this study, the lower dose levels (0.005% and 0.01%) of EC-12 did not prevent ED. Presently used EC-12 had a high cell pellet density of at least 5 trillion cells/g [15]. Therefore, the diet offered to pigs of E-0.05 group contained 2.5×10^9 *E. faecalis* EC-12 cells/g diet. Diets offered to pigs in other groups such as E-0.01 and E-0.005 contained 5.0×10^8 and 2.5×10^8 cells/g, respectively. The loss of preventive effect against ED at the lower dose levels of EC-12 can be explained at least partly by the steric hindrance to the attachement of STEC on the mucosal surface. From the present study, in addition to the preliminary field research, the effective dose-level of EC-12 was suggested to be 0.05% (w/w).

It is noteworthy, eosinophil infiltration was observed not only in STEC infected pigs but in healthy pigs. Eosinophil infiltration is a well-known allergic inflammation response occurring in patients with food allergies and asthma; this infiltration may contribute to tissue damage and connective tissue remodeling [1, 11]. Therefore, pigs may have an allergic symptom in the small intestine even in the healthy pigs.

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