

Use of a Trial Probiotic Product in Calves Experimentally Infected with *Escherichia coli* O157

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Abstract

It has been recognized that cattle are the main source of *Escherichia coli* O157:H7. This organism appears to be confined to the gastrointestinal tract and is shed in the feces. A probiotic product containing lactic acid-producing *Streptococcus bovis* LCB6 and *Lactobacillus gallinarum* LCB12 isolated from adult cattle was developed, and a preliminary experiment was conducted to evaluate its effect on the elimination of *E. coli* O157 from experimentally infected calves. Eight 4-month-old Holstein calves were orally challenged with *E. coli* O157 and the probiotic product was administered to 4 calves with continuous fecal shedding of *E. coli* O157 at 7 days after the infection. Fecal shedding of *E. coli* O157 was completely inhibited and re-shedding was not detected in any of the animals. Remarkable increase of the concentration of volatile fatty acids (VFAs), especially acetic acid in the feces after the administration of probiotic bacteria was correlated with the decrease in the number of *E. coli* O157. In the 4 calves in which fecal shedding of *E. coli* O157 had spontaneously stopped by the 7th day, a high concentration of VFAs was detected in the feces before and after the experimental infection. Although our results are preliminary and obtained from calves under limited conditions, the possibility of using a probiotic product to reduce fecal shedding of *E. coli* O157 from cattle was suggested.

Discipline: Animal health

Additional key words: bovine, volatile fatty acids

Introduction

Escherichia coli O157 was first recognized to be associated with human disease during an investigation of 2 outbreaks of hemorrhagic colitis in 1982²². Cattle, especially young animals, have been implicated as the principal source of *E. coli* O157:H7^{3,10,19,23}. A recent national survey conducted by the Ministry of Agriculture, Forestry and Fisheries of Japan revealed that 0.62% of cattle in randomly selected farms shed *E. coli* O157 in the feces²⁰. Additionally, prevalence levels of shedding of *E. coli* O157 in Scottish beef cattle aged between 12 and 30 months were reported in 8.6% of the animals, with 23.7% of the herds being infected²¹.

In experimentally infected cattle, the organism appears to be confined to the gastrointestinal tract and is shed in the feces^{2,4}. Management practices, such as high level of grain feeding⁷, fasting⁵, and transportation stress, are critical factors that are likely to affect the increase in the shedding of coliforms, including *E. coli* O157:H7. During the slaughtering and processing opera-

tions, meat surfaces may become contaminated by ingesta or feces on the hide¹². Two interrelated strategies to reduce the levels of *E. coli* O157:H7 organisms in slaughterhouses have been proposed⁵. One is to reduce the number of cattle shedding *E. coli* O157:H7, the other is to reduce the magnitude of shedding (CFU/g) by the animals infected with the organism. Presently, the improvement of the slaughtering and processing procedures (e.g. by the implementation of hazard analysis and critical control point-type programs and by the use of scientifically based critical management) is the most effective strategy. The development of methods for reducing or eliminating the carriage of *E. coli* O157:H7 in cattle on farms is essential to decrease the level of exposure to the pathogen via food and the environment. It is virtually impossible to use antibiotics against carrier animals due to the problem of residues and the possible selection of resistant bacteria.

The objective of this study was to describe the relationship between the concentration of fecal volatile fatty acids (VFAs) and colonization of *E. coli* O157 in experimentally infected calves. In the course of the study, a

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trial probiotic product was administered, and its efficacy in reducing or eliminating fecal shedding of *E. coli* O157:H7 from cattle was evaluated.

Experimental procedures

1) Animals and experimental infection

The challenge organism was a nalidixic acid- and rifampicin-resistant derivative of the *Escherichia coli* O157:H7 strain MN157 (*E. coli* MN157, *eaeA* and *stx2*-positive), an isolate from a calf with diarrhea. The stable colonization of this challenge strain in experimentally infected calves had already been confirmed in our previous report¹⁸⁾, indicating that the calves infected with the *E. coli* MN157 strain showed continuous fecal shedding of the organism for at least 5 weeks (Fig. 1). Eight 4-month-old Holstein calves were orally challenged with $10^{10.11}$ CFU of *E. coli* MN157. The calves were housed in segregated barns in accordance with the guidelines of animal experimentation defined by the National Institute of Animal Health (Japan). The pens had an individual floor drain and were cleaned twice daily. Calves with a similar size (ca. 100 kg) were fed twice a day (4 kg of dry matter per day) and had free access to water. The feed used was a mixture of 50% cut rice plant straw and 50% grain. In all the animals, culture for *E. coli* O157:H7 was negative prior to the challenge.

2) Recovery of challenge organism

After the challenge, the feces were collected on selected days directly from the rectum at 9:00 am before feeding. For direct plating, 2 g of feces were added to 18 mL of diluent and homogenized with Lab-Blender 80 (Seward Laboratory UAC House, London, England). A series of 10-fold dilutions was prepared and 0.1 mL of each dilution was spread on NRSMAC (sorbitol MacConkey agar [Eiken Chemical Co., Ltd., Tokyo, Japan] containing nalidixic acid [20 µg/mL] and rifampicin [20

µg/mL]). At the same time, 2 mL of each 10^{-1} dilution was spread on 5 well-dried NRSMAC plates. The limit for the detection of *E. coli* MN157 by the direct plating method described above was 1 CFU/g of feces. Additionally, the MPN method¹⁵⁾ was employed to detect shedding at levels lower than those detectable by the direct plating count. Samples of 10, 1, and 0.1 g of feces were added to 100 mL, 10 mL, and 10 mL of mEC broth (Eiken Chemical Co., Ltd.), respectively. Three sets of enrichment culture were prepared. After enrichment overnight at 37°C, 0.1 mL of each enrichment culture was spread on NRSMAC agar. The detection limit was 3 CFU of *E. coli* MN157/100 g of feces with this MPN method. The presence of *E. coli* MN157 on the NRSMAC plate was tested after overnight incubation at 37°C. Selected sorbitol-negative colonies typical of *E. coli* O157:H7 were tested for the O157 antigen by the slide agglutination test.

3) Determination of VFAs and lactic acid

The concentrations of VFAs and lactic acid in the fecal samples were determined by the method described in the Anaerobe Laboratory Manual¹⁴⁾.

4) Selection of probiotic bacteria

The isolation of potential probiotic bacteria was attempted in the feces of adult cattle. Based on the evidence that fecal shedding of *E. coli* O157:H7 persists longer in calves than in adult animals⁴⁾, the presence of bacteria that inhibit the colonization of *E. coli* O157:H7 was suspected in the feces of adult cattle. First, we investigated lactic acid-producing bacteria as candidates. Feces taken from the rectum of three 3-year-old adult cows and three 4-month-old calves were serially diluted 10-fold with an anaerobic diluent and inoculated on BL (blood liver) agar (Eiken Chemical Co., Ltd.) and LBS agar (Becton Dickinson Microbiology Systems, Cockeysville, MD). The use of BL agar is known to enable to detect accurately colony characteristics of anaerobic bacteria, especially lactobacilli, in particular for non-selective culture and comparison of intestinal microflora^{16, 17)}. After incubation for 3 days at 37°C under anaerobic conditions, bacterial colonies producing a sufficient amount of acid were enumerated and isolated. The population of lactic acid-producing bacteria in the feces of adult cows and calves was compared. In the course of the investigation, strains LCB6 and LCB12, the dominant lactic acid-producing bacteria in the feces of adult cows, were selected for further investigation. These 2 strains were subjected to detailed bacteriological identification based on biochemical tests and comparison of full-length 16S rRNA sequences. Biochemical tests were conducted

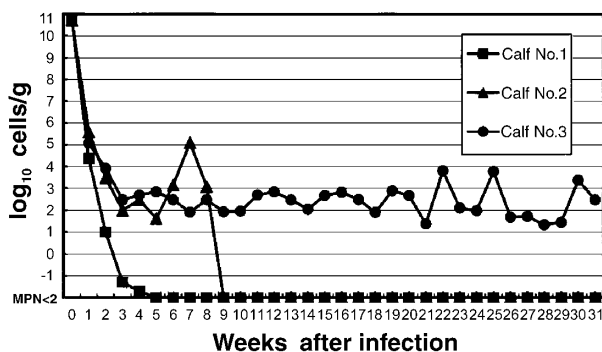


Fig. 1. Time course of fecal shedding of *E. coli* O157:H7 in experimentally infected calves

using an API Strep 20, API 50 CH, and API 50 CHL Kit (bioMérieux, Marcy l'Etoile, France). Comparison of full-length 16S rRNA sequences was entrusted to MIDI LABS (Delaware, U. S. A.).

5) Trial probiotic product

In preliminary experiments conducted to evaluate the efficacy of the strains LCB6 and LCB12, 10^9 CFU and 10^{11} CFU/head of fresh culture of these strains were administered for 3 days to 2 calves experimentally infected with *E. coli* O157:H7. Elimination of *E. coli* O157:H7 from the feces was ascertained in a calf which received 10^{11} CFU, and transitory decrease of the number of organisms was observed in another calf which received 10^9 CFU (data not shown). The dosage of probiotic bacteria for further experiments was fixed at 10^{11} CFU/head. The freeze-dried product of *S. bovis* LCB6 and *L. gallinarum* LCB12 mixed with dextrin as a preservative was prepared, and the concentration of each strain was adjusted to 10^{10} CFU/g. The product was kept at -20°C until use. The trial probiotic product was produced and supplied by courtesy of CALPIS Co., Ltd.

6) Administration and evaluation of the trial probiotic product

The probiotic product was administered to 4 calves (Nos. 1–4) with continuous fecal shedding of *E. coli* MN157 at 7 days after the infection. Twenty gram/head of freeze-dried product of probiotic bacteria mixed with grain feed was administered twice a day. The probiotic diet was administered during one week (from the 7 to 13th day) and another week (from the 17 to 23rd day) after a 3-day interruption. Determination of the concentrations of fecal VFAs and lactic acid, and enumeration of coliforms, *S. bovis* LCB6 and *L. gallinarum* LCB12, in the fecal samples were conducted on selected days. Coliforms were enumerated by inoculation with an appropriate dilution of feces on DHL (desoxycholate hydrogen sulfide lactose) agar (Eiken Chemical Co., Ltd.). The number of *S. bovis* LCB6 and *L. gallinarum* LCB12 in the fecal samples was counted using BL agar and LBS agar in the same way. The limit of detection of *S. bovis* LCB6 was 10^4 CFU/g of feces on BL agar, and that of *L. gallinarum* LCB12 was 10^2 CFU/g on LBS agar. A two-fold dilution of the fecal samples was prepared by the addition of distilled water, and the pH was measured with a combination electrode.

7) Use of the trial probiotic product in carrier cattle on-farm

An on-farm study was conducted on fattener herds of Japanese Black cattle 10 months of age. Prevalence

level of fecal shedding of *E. coli* O157 was approximately 80% in that farm. The trial probiotic product was administered to 18 cattle for 2 weeks and the rate of fecal shedding was compared with that of untreated 14 cattle on selected days.

Results and discussion

Strains LCB6 and LCB12 were identified as *Streptococcus bovis* and *Lactobacillus gallinarum*⁹⁾ based on biochemical tests and confirmation by comparison of full-length 16S rRNA sequences. Sequence searches of the EMBL/GenBank database using the FASTA program revealed that the newly determined sequences of strains LCB6 and LCB12 were most closely related to *Streptococcus bovis* (16S rRNA sequence similarities = 99%) and *Lactobacillus gallinarum* (16S rRNA sequence similarities = 100%), respectively.

In all the animals, fecal shedding of *E. coli* MN157 was observed on the day after the oral challenge. The calves did not show any clinical signs and remained healthy throughout the experiment. By the 7th day, the 4 animals (Nos. 1–4) that had shown a continuous shedding were subjected to an experiment to evaluate the efficacy of the probiotic product (Fig. 2). In 3 calves, fecal shedding of *E. coli* MN157 was no longer detectable on the 14th day at the end of the administration of the initial probiotic diet. One remaining calf (No. 2) continued shedding a small amount of *E. coli* MN157, ranging from 43 to 500 CFU/100 g of feces, after the first period of probiotic administration. Four calves were fed another series of the probiotic product from the 17th to the 23rd day. The shedding of *E. coli* MN157 from calf No. 2 had ceased by the 28th day, and re-shedding was not detected in any of the animals in a follow-up survey for 3 consecutive weeks. Fecal shedding of *E. coli* MN157 was completely inhibited by the administration of the freeze-dried product of probiotic bacteria isolated from the feces of the adult cows. All the calves were clinically normal throughout the experiment.

On the whole, an increase of 1 to 3 \log_{10}/g in the numbers of *S. bovis*, *L. gallinarum*, and coliforms was observed during the period of probiotic feeding. In contrast to this increase, fecal shedding of *E. coli* MN157 decreased quickly. In 3 calves (except for No. 4), fecal pH in the range of 6 to 7 was maintained throughout the experiment. Calf No. 4 showed a lower pH value of around 6, which increased temporarily to 8 during the first period of probiotic feeding. No significant change of pH correlated with the decrease of the number of *E. coli* MN157 was observed. The most remarkable change recognized consistently in all the 4 calves (Nos. 1–4) was a

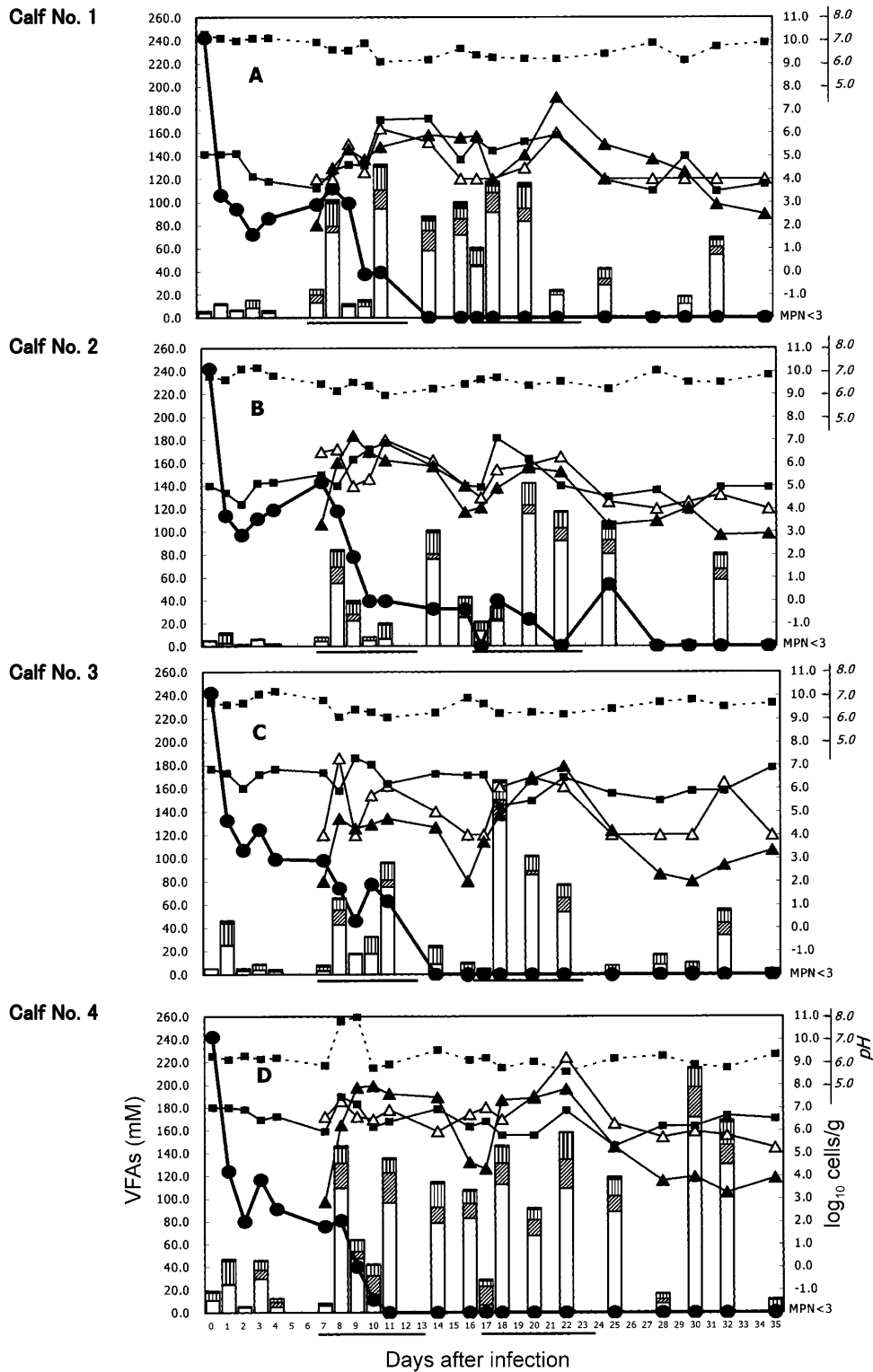


Fig. 2. Elimination of fecal shedding of *E. coli* O157:H7 in infected calves by feeding of the probiotic product

Acetic acid,
 Propionic acid,
 Butyric acid,
 Valeric acid: VFAs (mM).
 —△— LCB6, —▲— LCB12, —■— Coliforms, —●— O157: Bacterial counts (\log_{10} cell/g).
 - - - ■ - - - : pH.
 Underlined days indicate the period of probiotic feeding.

Table 1. Mean concentration (mM) of total VFAs and respective acids in the feces of calves before the administration and over the feeding period of the probiotic product

Calf No.	Before administration (days 0–7)					Feeding period (days 8–23)				
	VFAs	Acetic acid	Propionic acid	Butyric acid	Valeric acid	VFAs	Acetic acid	Propionic acid	Butyric acid	Valeric acid
1	11.8	7.6	1.2	2.5	0.4	76.8 ^{b)}	55.4 ^{b)}	8.2 ^{a)}	10.2 ^{c)}	2.9 ^{c)}
2	5.8	3.4	0.2	1.7	0.5	61.2 ^{b)}	43.2 ^{a)}	5.0 ^{a)}	11.5 ^{c)}	1.5 ^{a)}
3	12.9	7.0	0.3	4.6	1.0	59.8 ^{a)}	43.9 ^{a)}	5.3 ^{a)}	9.4	1.3
4	22.9	13.3	2.3	6.3	1.0	103.6 ^{c)}	71.7 ^{b)}	18.0 ^{c)}	12.2	1.8
5	88.8	60.1	14.8	11.7	2.3	No determination				
6	41.9	30.3	6.4	3.6	1.7	No determination				
7	67.9	40.3	14.6	11.4	1.6	No determination				
8	84.6	57.3	15.2	10.3	1.9	No determination				

a): $P < 0.05$, b): $P < 0.01$, c): $P < 0.001$ (two sample t tests with Welch's correction). Value statistically significantly different from corresponding value.

considerable increase of the concentration of VFAs in the feces after the initial administration of probiotic bacteria. The increase of the concentration of VFAs was most obvious on the 8th and 18th day, the day after the administration of probiotic feeding. The mean concentration (mM) of total VFAs and that of the respective acids in the feces in each animal were significantly different between the days before the administration and over the feeding period of the probiotic product (Table 1). Four calves (Nos. 5–8) in which spontaneous fecal shedding of *E. coli* MN157 had stopped by the 7th day, exhibited a high concentration of VFAs in the feces before and after the experimental infection (Table 1).

In the on-farm study, the averaged fecal shedding rates of *E. coli* O157 in the probiotic-treated and untreated groups throughout the experiment were 77.5 and 80.0%, respectively. No significant decrease of fecal shedding of *E. coli* O157 was observed by the administration of the trial probiotic product.

In the present study, it was suggested that the concentration of VFAs in the feces might be associated with the presence of *E. coli* O157:H7 in cattle. In calf No. 2 which continued to show a prolonged fecal shedding of *E. coli* MN157, the total amount of fecal VFAs during the first administration of the probiotic product was smaller than that in other 3 calves. It has been demonstrated that VFAs are toxic to Gram-negative bacteria¹³⁾. Bohnhoff et al.¹⁾ reported that the growth of *Salmonella* was inhibited by the addition of acetic and/or butyric acid in broth culture, while lactic acid acted antagonistically with these acids. About 70% of the VFAs detected in the cattle feces in this experiment consisted of acetic acid. Bacteriostatic or bacteriocidal activity of acetic acid on food-borne pathogenic bacteria including *E. coli* O157:H7, has

received a great deal of attention. The growth of all the strains of *E. coli* O157:H7 tested was inhibited by a 0.1% (ca. 17.5 mM) concentration of acetic acid *in vitro*⁸⁾. Much higher concentrations of acetic acid may be necessary to induce a bacteriostatic or bacteriocidal activity in the feces of animals infected with *E. coli* O157:H7.

The probiotic bacterial strains used in this experiment produced a sufficient amount of lactic acid but only a small amount of acetic acid. Lactic acid was detected at a low concentration (below 1 mM) in the fecal samples throughout the course of the experiment. Some kinds of bacteria act antagonistically against *E. coli* O157:H7 by producing a large amount of acetic acid, and the concentration of other VFAs might have increased immediately in response to the administration of the probiotic bacteria. A study aimed at culturing the bacteria which might have been responsible for the rapid increase of the concentration of fecal VFAs was conducted using fecal samples stored at -80°C . A remarkable increase of more than 2 \log_{10}/g in the number of *Bifidobacterium* species was confirmed in calves No. 3 and No. 4 on the 8th day while no detectable changes in the number of bacterial populations were observed in the other 2 animals (data not shown).

Characterization of these bacteria and further studies may contribute to the elucidation of the mechanisms of elimination of *E. coli* O157:H7 from the gastrointestinal tract of cattle. From the onset, this experiment was conducted based on the hypothesis that the administration of a large amount of probiotic bacteria would alter the ecology of the intestinal microflora and lead to a reduction of the number of coliforms, including *E. coli* O157:H7. An inverse relationship, however, between the population of coliforms and *E. coli* O157:H7 was recognized. It was

reported that a fresh culture of 17 strains of *E. coli* and one strain of *Proteus mirabilis* administered to cattle prior to exposure to *E. coli* O157:H7 could reduce the level of carriage of the organism in most animals²⁴. Localization of these bacteria at the same sites of the mucosal surface of the intestine as *E. coli* O157:H7 and the production of some kinds of metabolites which inhibit the target bacteria have been postulated as possible mechanisms. The present study indicates that direct or indirect microbial interactions provide a useful means to reduce or eliminate enteric pathogens from the animal gastrointestinal tract.

Although a real control group, namely, animals infected with *E. coli* O157 but not treated with the probiotic product was not used in this experiment, in a previous report¹⁸, it was shown that in calves infected with *E. coli* MN157 strain, fecal shedding of the organism continued for at least 5 weeks. The treatment of cattle with a probiotic product was found to eliminate fecal shedding of *E. coli* O157:H7 in experimentally infected calves. However, no significant decrease of fecal shedding of the organism was detected in the on-farm study.

Many factors such as the feeding content, breed, age, and the husbandry conditions of the cattle that influence *E. coli* O157 infection prevalence have been examined^{6,7,11}. Although our results are preliminary and obtained from calves under limited conditions, further studies will be conducted to confirm the efficacy of the probiotic product in cattle carrying *E. coli* O157:H7 in farms.

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