Significance of Fecal Volatile Fatty Acids in Shedding of *Escherichia coli* O157 from Calves: Experimental Infection and Preliminary Use of a Probiotic Product

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ABSTRACT. Cattle have been recognized as a principal reservoir of *Escherichia coli* O157:H7. This organism appears to be confined to the gastrointestinal tract and is shed in 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 against four calves continued fecal shedding of *E. coli* O157 by the 7th day after infection. Fecal shedding of *E. coli* O157 was completely inhibited and re-shedding was not detected in any of the animals. Remarkable increase of VFAs, especially that of acetic acid in feces after the administration of probiotic bacteria correlated with the diminution of *E. coli* O157. Four calves that had spontaneously ceased fecal shedding of *E. coli* O157 by the 7th day exhibited a high concentration of VFAs in feces before and after experimental infection. Although our results are preliminary and obtained from calves under limited conditions, the possible application of probiotic product to reduce fecal shedding of *E. coli* O157 from cattle is suggested.

KEY WORDS: bovine, *Escherichia coli* O157, probiotic, volatile fatty acid.

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Escherichia coli O157 was first associated with human disease during an investigation of two outbreaks of hemorrhagic colitis in 1982 [21]. Cattle, especially young animals, have been implicated as a principal reservoir of *E. coli* O157:H7 [3, 10, 20, 22]. In experimentally infected cattle, the organism appears to be confined to the gastrointestinal tract and is shed in feces [2, 4]. Management practices, such as high proportional grain feeding [7], fasting [5], and transportation stress, are critical factors likely to affect an increase in the shedding of coliforms, including *E. coli* O157:H7. During slaughter and processing, meat surfaces may become contaminated by ingesta or feces on the hide [12]. Two inter-related strategies to reduce the levels of *E. coli* O157:H7 organisms in slaughter plants have been proposed [5]. 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 those animals infected with the organism. At present, the improvement of slaughtering and processing procedures (e.g. by employing hazard analysis and critical control point-type programs and by using scientifically based critical management) is the most practical strategy. Methods for reducing or eliminating the carriage of *E. coli* O157:H7 in cattle on farms are 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 purpose of this study was to describe the relation between fecal volatile acids (VFAs) concentration and colonization of *E. coli* O157 in experimentally infected calves. In the course of the study, administration of a trial probiotic product was conducted, and its efficacy in reducing or eliminating fecal shedding of *E. coli* O157:H7 from cattle was

evaluated.

MATERIALS AND METHODS

Animals and experimental infection: The challenge organism was nalidixic acid and a 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. 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 of 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. All animals were culture negative for *E. coli* O157:H7 prior to challenge.

Recovery of challenge organism: After the challenge, feces were collected on selected days directly from the rectum at 9:00 am before feeding. For direct plating, 2.0 g of feces was added to 18.0 ml of diluent and homogenized with Lab-Blender 80 (Seward Laboratory UAC House, London, England). A serial 10-fold dilution was made 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, two ml of each 10^{-1} dilution was spread on five well-dried NRSMAC plates. The limit for detection of *E. coli* MN157 by the direct plating method described above was 1 CFU/g of feces. Additionally, the MPN method [15] was

employed to detect shedding at levels lower than those detectable by a direct plating count. Samples of 10, 1.0, 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. A limit of three CFU of *E. coli* MN157/100 g of feces was detectable 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.

Determination of VFA and lactic acid: The concentration of volatile fatty acids (VFAs) and lactic acid in the fecal samples were determined by the method described in the Anaerobe Laboratory Manual [14].

Selection of probiotic bacteria: The isolation of potential probiotic bacteria was attempted with the feces of adult cattle. Based on the evidence that fecal shedding of *E. coli* O157:H7 persists longer in calves than adults [4], 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. Rectum feces taken from three 3-year-old adult cows and three 4-month-old calves were serially diluted 10-fold with anaerobic diluent and inoculated on BL (blood liver) agar (Eiken Chemical Co., Ltd.) and LBS agar (Becton Dickinson Microbiology Systems, Cockeysville, MD). BL agar is known to clearly reflect colony characteristics of anaerobic bacteria, especially lactobacilli, and it is frequently used for non-selective cultivation 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 research. These two strains were subjected to precise bacteriological identification by biochemical tests and comparison of partial 16S rRNA sequences. Biochemical tests were conducted using an API Strep 20, API 50 CH, and API 50 CHL Kit (bioMérieux, Marcy l'Etoile, France). To compare the phylogenetic similarities of strains LCB6 and LCB12, their 16S rRNA genes were amplified by PCR using the primer pair pL (5'-AGAGTTTGATCCTGGCTCAG-3') and pR (5'-CTACTGCTGCCTCC CGTAG-3'), based on the 16S rRNA sequence [18] and subjected to sequence analysis. About 300 bp of sequences of these two strains corresponding to upstreams of the 16S rRNA gene sequence were determined, and comparative sequence analysis was conducted.

A trial probiotic product: In preliminary experiments to evaluate the efficacy of the strains LCB6 and LCB12, 10^9 CFU and 10^{11} CFU per head of fresh culture of these strains were administered for 3 days to two calves experimentally infected with *E. coli* O157:H7. Elimination of *E. coli*

O157:H7 from feces was ascertained in a calf dosed with 10^{11} CFU, and a temporal decrease of the organism was observed in another calf dosed with 10^9 CFU (data not shown). The dosage of probiotic bacteria for further experiments was determined 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.

Administration and evaluation of the probiotic product: The probiotic product was administered against four calves (Nos. 1–4) continued fecal shedding of *E. coli* MN157 by the 7th day after infection. Twenty grams per head of freeze-dried product of probiotic bacteria mixed with grain feed was administered twice a day. The probiotic diet was conducted for a week (from the 7 to 13th day) and another one week (from the 17 to 23rd day) after 3 days break. Determination of fecal VFA and lactic acid, enumeration of coliforms, *S. bovis* LCB6 and *L. gallinarum* LCB12 in 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 sample was counted using BL agar and LBS agar in the same manner. 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 made by the addition of distilled water, and the pH was measured with a combination electrode.

RESULTS

Strains LCB6 and LCB12 were identified as *Streptococcus bovis* and *Lactobacillus gallinarum* [9] by biochemical tests and confirmed by comparison of partial 16S rRNA sequences. Sequence searches of the EMBL/GenBank database using the FASTA program revealed that newly determined sequences of strains LCB6 and LCB12 were most closely related to *Streptococcus bovis* (16S rRNA sequence similarities >99.41%) and *Lactobacillus gallinarum* (16S rRNA sequence similarities >99.39%), respectively.

All animals began detectable fecal shedding of *E. coli* MN157 the day after the oral challenge. The subjected calves did not show any clinical signs and remained healthy throughout the experiment. By the 7th day, the four animals (Nos. 1–4) that had continued shedding were subjected to an experiment to evaluate the efficacy of the probiotic product (Fig. 1). Three calves had ceased detectable fecal shedding of *E. coli* MN157 by the 14th day at the end 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 during the

Fig. 1. Elimination of fecal shedding of *E. coli* O157:H7 in infected calves by feeding of the probiotic product and other parameters: bacterial counts of *S. bovis* LCB6, *L. gallinarum* LCB12, and coliforms; measured values of pH; and VFAs (acetic, propionic, butyric, and valeric acid). (A), calf No. 1; (B), calf No. 2; (C), calf No. 3; (D), calf No. 4. Underlined days indicate the period of probiotics feeding.

course of a follow-up survey which continued 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 calves were clinically normal throughout the experiment.

On the whole, increases of 1 to 3 $\log_{10}/g$ of *S. bovis* LCB6, *L. gallinarum* LCB12, and coliforms were observed during the period of probiotic feeding. In contrast to these increases,

fecal shedding of *E. coli* MN157 decreased quickly. In three calves (excepting No. 4), fecal pH in the range of 6 to 7 was maintained throughout the experiment. Calf No. 4 showed a lower pH of around 6, which increased temporarily to 8 during the first period of probiotic feeding. No significant change of pH correlating with the diminution of *E. coli* MN157 was observed. The most surprising change recognized consistently in all four calves (Nos. 1–4) was a remarkable increase of VFAs in feces after the initial administration of probiotic bacteria. The increase of VFAs was most obvious on the 8th and 18th day, the day after administration of probiotic feeding. The mean concentration (mM) of total VFAs and that of respective acids in the feces of each animal were significantly different between the days before administration and over the feeding period of the probiotic product (Table 1). Four calves (Nos. 5–8) that had spontaneously ceased fecal shedding of *E. coli* MN157 by the 7th day exhibited a high concentration of VFAs in feces before and after experimental infection (Table 1).

DISCUSSION

In the present study, it was suggested that the concentration of VFAs in feces might be associated with the presence of *E. coli* O157:H7 in cattle. Calf No. 2 which continued a longer fecal shedding of *E. coli* MN157, had a smaller total amount of fecal VFAs during the first administration of the probiotic product than the other three calves. It has been known that VFAs are toxic for Gram-negative bacteria [13]. Bohnhoff *et al.* [1] demonstrated that the growth of *Salmonella* was inhibited by the addition of acetic and/or butyric acid in broth culture, otherwise lactic acid acted antagonistically with these acids. About 70% of the VFAs detected in cattle feces in this experiment were acetic acid. Bacteriostatic or bacteriocidal activity of acetic acid on food-borne pathogenic bacteria including *E. coli* O157:H7 has received considerable attention. The growth of all 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 bacteriostatic or bacteriocidal activity in the feces of *E. coli* O157:H7 infected animals.

The probiotic bacteria strains used in this experiment produce 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 fecal samples throughout the course of the experiment. Some kind of bacteria act antagonistically against *E. coli* O157:H7 by producing a large amount of acetic acid, and other VFAs might have increased immediately in response to the administration of the probiotic bacteria. The isolation and characterization of those bacteria would 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 administration of a large amount of probiotic bacteria would alter the ecology of intestinal microflora and lead to the diminution of coliforms,

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

Calf No.	Before administration (days 0–7)					Feeding period (days 8–23)				
	VFAs	Acetic	Propionic	Butyric	Valeric	VFAs	Acetic	Propionic	Butyric	Valeric
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	Not done				
6	41.9	30.3	6.4	3.6	1.7	Not done				
7	67.9	40.3	14.6	11.4	1.6	Not done				
8	84.6	57.3	15.2	10.3	1.9	Not done				

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

including *E. coli* O157:H7. An inverse relationship, however, between the population of coliforms and *E. coli* O157:H7 was recognized. It is 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 can reduce the level of carriage of the organism in most animals [23]. Localization of these bacteria at the same sites of mucosal surface of the intestine as *E. coli* O157:H7 and the production of some kinds of metabolites which inhibit the target bacteria are 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.

The real control group, namely, animals infected with *E. coli* O157 but not treated with the probiotic product was not involved in this experiment, however our previous report [19] provides a redeeming data indicating that the calves infected with *E. coli* MN157 strain continued fecal shedding of the organism for at least five weeks. The treatment of cattle with a probiotic product was shown to eliminate fecal shedding of *E. coli* O157:H7 in experimentally infected calves. This finding may contribute to a reduction or elimination of environmental contamination from this pathogen. Many factors such as the feeding content, breed, age, and the husbandry condition of the cattle that influence *E. coli* O157 infection prevalence have been discussed [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 against cattle carrying *E. coli* O157:H7 on farms.

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