Inhibitory Effects of *Bifidobacterium longum* on Enterohemorrhagic *Escherichia coli* O157:H7

Kyoko NAMBA,*1 Tomoko YAESHIMA,1 Norio ISHIBASHI,1 Hirotoshi HAYASAWA1 and Shoji YAMAZAKI2

1Nutritional Science Laboratory, Morinaga Milk Industry, Co., Ltd., 5–1–83, Higashihara, Zama 228–8583, Japan
2Department of Veterinary Public Health, National Institute of Public Health, 4–6–1, Shiroganedai, Minato-ku, Tokyo 108–8638, Japan

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The effects of *Bifidobacterium longum* BB536 (BB536) on *Escherichia coli* O157:H7 IPH9 (IPH9) were first examined using germ-free mice mono-associated with BB536 (BB536-MA). Thirty-two days after oral challenge with a lethal dose of IPH9, all the non-associated germ-free mice (GF) died, but all the BB536-MA mice survived and the number of IPH9 in the feces was lower than that in GF mice. Second, co-culture experiments with BB536 and IPH9 were performed. The number of IPH9 in the co-culture was less than 1% of that in mono-culture after 24 hr, and the verotoxin concentration also decreased. Culture of IPH9 under various conditions showed that lactate, acetate and the supernatant of BB536 culture had anti-O157 activities even when the pH was neutral, and the supernatant of BB536 culture had stronger inhibitory effects than lactate or acetate against the production of verotoxin. This efficient inhibition of verotoxin may suggest the presence of verotoxin-inhibitory factors in BB536 metabolites in addition to lactate and acetate.

Key words: *Escherichia coli* O157:H7; Bidifodobacterium; probiotics; gnotobiotic mice; verotoxins

INTRODUCTION

Bifidobacteria are now almost as commonly used as lactobacilli in yogurts and other dairy products, and they are also attractive as probiotics for humans since they are one of the predominant microorganisms in intestinal microflora. Especially in breast-fed infants, bifidobacteria always exist as the predominant bacteria in the fecal microflora and are thought to be a protective factor against diarrhea. *Bifidobacterium longum* is one of the *Bifidobacterium* species found mainly in human feces, and has been most often used industrially. *B. longum* strain BB536 was isolated from the feces of a healthy baby in 1969. Many physiological effects and protective effects against pathogens have been reported, and the strain has been used commercially for various food applications in several countries.

*Escherichia coli* O157:H7 was first reported in America in 1982. Since then, many large outbreaks of *E. coli* O157-infection have occurred around the world. *E. coli* O157 is known to be exceptionally tolerant of acidic pH, and to survive in gastric juice. The serotype O157:H7 generally produces verotoxins and has been identified as a causative agent of diarrhea and hemolytic uremic syndrome (HUS). Recently, many reports have described the antagonistic actions of *Lactobacillus, Bifidobacterium* and fermented milk on pathogenic *E. coli* and *B. infantis*. In animal studies, it has been demonstrated that bifidobacteria are capable of enhancing immunity and preventing invasion of pathogenic *E. coli*. Antibacterial substances produced by bifidobacteria were also reported in some *in vitro* studies. Acetic acid and lactic acid seem to be the most important factor. However, there is a report of other inhibitory compounds produced by *B. infantis*. In that report, the most active fraction of bifidobacterial extracts which contains no acetate or lactate strongly inhibited the growth of pathogenic *E. coli*.

In previous studies, we found that monoassociation with BB536 before infection can enhance immune function in germ-free mice, and significantly resist translocation of pathogenic *E. coli* O111. We also reported the inhibitory effects of BB536 on harmful bacteria including pathogenic *E. coli* O111 in *vitro*. In this study, we investigated the effect of BB536 in protection against *E. coli* O157-infection in *vivo* using BB536-monoassociated mice, and also conducted some *in vitro* experiments to elucidate the inhibitory factors.

MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *B. longum* BB536 was preserved and maintained in the culture collection of Morinaga Milk Industry Co., Ltd. Subculture was performed once a week in GAM broth.
(Nissui, Japan) plus 1.7% (w/v) glucose and incubated anaerobically and statically for 16 hr at 37°C. An anaerobic jar (GasPack, BBL, USA) with disposable O2 absorbing and CO2 generating agent (AnaeroPack, Mitsubishi Gas Chemical, Japan) was used for anaerobic conditions. The enterohemorrhagic Escherichia coli O157:H7 IPH9 that produces verotoxin 1 (VT1) and verotoxin 2 (VT2) was isolated from a patient in Japan. Subculture was performed once a week in LB broth (1% Difco tryptone, 0.5% Difco Yeast extract, 1% NaCl, pH 7.2–7.4) and incubated aerobically and statically for 16 hr at 37°C. Pre-cultivation of BB536 and IPH9 was performed in the control media for each experiment described later, and all of the in vitro experiments were performed under static conditions at 37°C.

Mice. Male and female germ-free (GF) and specific pathogen-free mice (SPF) of BALB/c background were used at 8–12 weeks of ages. Methods of maintenance and checking of the germ-free environment in the isolator were as described previously (25). Germ-free mice monoassociated with BB536 (BB536-MA) were prepared by an oral administration of BB536 (10^7 per mouse) 29 days before IPH9-infection.

Measurement of viable bacterial numbers. In all experiments, viable bacterial numbers were determined by counting colony forming units (cfu) on Sorbitol MacConkey agar plates (Oxoid) for IPH9 and BL agar plates (Nissui, Japan) supplemented with 5% (v/v) horse blood for BB536. The samples including IPH9 or BB536 were inoculated onto the respective plates after appropriate dilutions with phosphate-buffered saline (PBS, pH 7.0). The plates of IPH9 and BB536 were incubated at 37°C for 16 hr under aerobic conditions and 72 hr under anaerobic conditions, respectively.

Measurement of verotoxins. In the in vitro experiments, the supernatants of the IPH9 culture containing extracellular verotoxin (VT2) were separated by centrifugation for 15 min at 3,000 × g, and the titer of each verotoxin was determined by a reversed passive latex agglutination kit (VTEC-RPLA “SEIKEN,” Denka Seiken, Japan) and expressed as RPLA titers.

Assays. Concentrations of lactic and acetic acid in the culture were assayed by the F-kit (Boehringer Mannheim, Germany), and the concentration of glucose was analyzed by Glucose B-test Wako (Wako Pure Chemical Industries, Japan).

Protective effects of BB536 against IPH9-infection. To investigate the protective effects of BB536 against IPH9-infection in germ-free mice, BB536-MA mice were tested in the experiment, and GF and SPF mice were used as controls. Eight mice per group were tested. IPH9 was administrated intragastrically (i.g.) into mice in the three groups described above at a high lethal dose (10^7 cfu per mouse). The survival rates in each group were observed for 42 days, and the viable numbers of IPH9 and BB536 per gram wet feces were measured at 0, 1, 4, 8, 14, 21 and 42 days after infection. At the end of the experiment, all the surviving mice were sacrificed and necropsied.

Inhibitory effects of BB536 on growth and toxin production of IPH9 by co-cultivation. To examine the effects of BB536 on the growth of IPH9 and the production of verotoxins, a 24-hr co-culture experiment was performed. Co-cultivation was performed by mixing BB536 and IPH9 at a ratio of approximately 1:1 (10^7 cfu ml^-1), and mono-cultivation of each strain was also included as a control. Before inoculation, BB536 and IPH9 cultures were centrifuged at 2,000 × g for 10 min, washed with PBS and resuspended in PBS. All cultivations were done in GAM broth plus 1.7% (w/v) glucose under anaerobic condition as described above. Samples were taken at 4 hr intervals for measurements of viable bacterial numbers, extracellular verotoxins and pH of the culture.

Stability of verotoxin under acidic conditions. The supernatant of the IPH9 culture containing extracellular verotoxin (VT2) was prepared by centrifugation described above, and sterilized by membrane filtration (0.22 µm Millex-GV, Millipore, USA). After adjusting to various pH values in the range of 4.0 to 7.0 using acetic acid, lactic acid or sodium hydroxide solution, the supernatants were incubated statically at 37°C for 24 hr and the concentrations of verotoxin were determined as RPLA titers.

Inhibitory effects of BB536-supernatant on growth and toxin production of IPH9. The supernatant of BB536 cultured in GAM broth plus 0.7% (w/v) glucose was prepared as for the supernatant of IPH9 culture described above, and neutralized with NaOH to pH 7.0. The glucose concentration of the supernatant was 0.0%. As controls, standard GAM broth (control 1) and GAM diluted two-fold with sterile water to lower the nutrition content (control 2) were used. The glucose concentrations in all the media was adjusted to 0.3% (w/v), which is the concentration used in the standard GAM broth and has been shown to have no adverse effect on the growth of IPH9 (5). Using BB536-supernatant and control media, IPH9 was cultivated aerobically for 24 hr, and the samples were taken at 0, 4, 8 and 24 hr for measurements of viable bacterial numbers, extracellular verotoxin (VT2) concentrations, and pH values.
Inhibitory effects of lactic and acetic acid on growth and toxin production of IPH9 in vitro. IPH9 was cultured in three types of medium. Lactic acid-supplemented medium and acetic acid-supplemented medium were prepared by adding 0.1 mol l⁻¹ lactic and acetic acid, respectively, to GAM broth followed by neutralization to pH 7.0 with NaOH (pH values before neutralization were 4.0 and 4.6, respectively; and the concentrations of acids were assayed by the F-kit). Acetate-supplemented medium was prepared by adding 0.1 mol l⁻¹ sodium acetate to GAM broth (pH 7.0). The culture samples were taken at 0, 4, 8 and 24 hr for measurements of viable bacterial numbers, extracellular verotoxin (VT2) concentrations, and pH values.

RESULTS

Protective Effects of BB536 against IPH9-Infection

As shown in Fig. 1, all GF mice infected with IPH9 died within 32 days of infection, but all of the BB536-MA mice survived up to the end of the experiment. The viable bacterial numbers in the feces are shown in Fig. 2. In GF mice, the number of IPH9 increased to $2 \times 10^{10}$ cfu g⁻¹ feces. In BB536-MA mice, the number of BB536 maintained high levels during the experimental period, but the growth of IPH9 was inhibited to 10% of the counts in the GF mice. In SPF mice, IPH9 was eliminated within a few days and all the mice were alive throughout the experiment. Diarrhea was not observed in any of the three groups. Both BB536-MA and SPF mice showed no potentially fatal abnormalities. The necropsy reports of SPF mice were normal, but some of the BB536-MA mice showed mild lesions in the kidney.

Inhibitory Effects of BB536 on Growth and Toxin Production of IPH9 by Co-Cultivation

Growth curves of the co-culture experiment are indicated in Fig. 3a. Twenty-four hours after co-cultivation with BB536, the number of IPH9 decreased to below 1% of that observed in IPH9 mono-cultivation, while the growth curves of BB536 were similar in both co-cultivation and BB536 mono-cultivation. The pH values were also similar in the co-culture and BB536 mono-culture (Fig. 3b). The verotoxin titers of the co-culture were obviously lower than the IPH9 mono-culture after 24 hr (Fig. 3c). The concentrations of lactic and acetic acid after 24 hr were 0.070 mol l⁻¹ and 0.109 mol l⁻¹ in BB536 mono-culture.

Stability of Verotoxin under Acidic Conditions

Figure 4 shows decreasing VT2 titers with time of incubation under acidic conditions. There were no significant differences between using lactic acid and acetic acid for pH adjustment; therefore only the data for acetic acid are shown. The titer of pH 7.0 did not decrease at 8 hr, but that of pH 4.0 decreased to 32%.

Inhibitory Effects of BB536-Supernatant on Growth and Toxin Production of IPH9

Figure 5 shows the viable numbers of IPH9 and concentrations of VT2 when IPH9 was cultured in BB536-supernatant as medium. The numbers of IPH9 were about 40% of control 1 from 4 to 24 hr of culture, but the titers of verotoxin were 5%, 10% and 12% at 4, 8
Fig. 3. Inhibition of E. coli O157:H7 IPH9 by co-cultivation with B. longum BB536.
(a) Growth curves of E. coli O157:H7 IPH9 (solid lines) in co-culture (○) and mono-culture (●); and growth of B. longum BB536 (broken lines) in co-culture (▲) and mono-culture (▲). (b) The pH values of E. coli O157:H7 IPH9 mono-culture (●), B. longum BB536 mono-culture (▲) and co-culture (○). (c) Concentrations of extracellular verotoxins (VT). VT1 (broken lines) in co-culture (○) and mono-culture (●); and VT2 (solid lines) in co-culture (○) and mono-culture (●) are shown. All results are mean values of duplicate experiments.

and 24 hr, respectively. The final culture pH values were all around 6.5 at 24 hr. The concentrations of lactic and acetic acid in the initial BB536-supernatant before neutralization were 0.039 mol l⁻¹ and 0.076 mol l⁻¹, and the pH was 4.4.

Inhibitory Effects of Lactic and Acetic Acid on Growth and Toxin Production of IPH9 in Vitro
The inhibitory effects of the addition of lactic and

Fig. 4. Stability of verotoxin (VT) under acidic conditions. VT2 titers at pH 7.0 (●), pH 6.0 (▲), pH 5.0 (▲), and pH 4.0 (□). All results are mean values of duplicate experiments.

Fig. 5. Inhibitory effects of B. longum BB536 culture supernatant on E. coli O157:H7 IPH9.
(a) Viable numbers of E. coli O157:H7 IPH9 (bars) cultured in B. longum BB536 culture supernatant (●), control 1 (undiluted GAM; ■), and control 2 (GAM diluted two-fold with sterile water; ▲); and pH (solid lines) of B. longum BB536 culture supernatant (○), control 1 (●), and control 2 (▲). (b) Concentrations of extracellular VT2 in B. longum BB536 culture supernatant (●), control 1 (■), and control 2 (▲). All results are mean values of triplicate experiments.
acetic acid to the culture medium on the growth of IPH9 and production of verotoxin are shown in Fig. 6. After incubation for 4 hr, the number of IPH9 and the titer of VT2 were 74% and 54%, respectively, of the control in the medium supplemented with 0.1 mol l⁻¹ lactic acid, and they were inhibited to 17% and 7% in the medium with 0.1 mol l⁻¹ acetic acid. At 24 hr, the number of IPH9 and the VT2 titer were 59% and 43%, respectively, of the control in the acetic acid-supplemented medium, but these values were both above 80% in the lactic acid-supplemented medium. The pH values of the culture were all approximately 6.5 after 24 hr. The inhibition by adding 0.1 mol l⁻¹ of sodium acetate is also shown in the same figure. Acetate-supplemented medium inhibited IPH9 almost as efficiently as the medium with added acetic acid followed by neutralization.

**DISCUSSION**

In this study, the inhibition of *E. coli* O157:H7 IPH9 by *B. longum* BB536 was first examined by an in vivo experiment using germ-free mice. All the BB536-MA mice survived but not the GF mice, and the intestinal proliferation of IPH9 in BB536-MA mice was suppressed. IPH9-infection was not established in SPF mice. These results imply that the intestinal microflora plays an important role in preventing O157-infection. A report of *B. adolescens* in mice by Oike et al. (19) almost completely agreed with our results, and these data together indicate that the antagonistic actions toward O157-infection may be common for *Bifidobacterium*-monoassociated mice. The immune response of BB536-monoassociated mice has been reported previously (26, 27). According to these studies, total IgA in secretory fluids and serum reached the levels observed in microflora-bearing mice (SPF) at 4 weeks after administration (i.g.) of BB536. Cell-mediated immune (CMI) response and the inhibition of bacterial translocation induced by BB536 started around the same time; moreover, the translocation of *E. coli* O111 was also inhibited after 4 weeks of BB536-monoassociation. In the present study, BB536 was administered about 4 weeks before IPH9-infection; therefore the level of immunocompetence in BB536-MA mice should be close to that in SPF mice. The activation of the host’s immune system by BB536 may be one of the contributing factors for survival in these mice. Additionally, recent reports have indicated that O157 inhabits the intestine by sticking to the intestinal wall (17). This may imply that monoassociation of BB536 before O157-infection obstructs the adherence of 0157 to the intestinal wall and also inhibits the passage of verotoxins into the bloodstream.

In the co-culture experiment, we found that BB536 inhibited the growth of IPH9 and decreased the extracellular verotoxins. The observation of a decrease in culture pH suggested that the inhibition might be caused by the acidic conditions (around pH 4.0 at 24 hr). Indeed, we found that the growth of IPH9 was inhibited by low pH (data not shown). This finding together with the result of instability of verotoxin under acidic conditions suggest that extracellular verotoxins in the coculture may also be denatured by the low pH.

The O157 cultivation experiment with lactic or acetic acid supplementation followed by neutralization or with sodium acetate supplementation also showed in-
hibitory effects. These results indicate that even at a neutral pH, lactate, acetate or non-dissociated acids may also act as inhibitory factors. Recently, many data on the acid resistant responses of O157 in the presence of various organic acids have been reported (6, 20, 22). In all these reports, hydrochloric acid has the least effect in inhibiting the growth of O157, and acetic or lactic acid is the most deleterious organic acid. We speculate that non-dissociated acids can easily cross the cell membrane (1) to lower the intracellular pH and inhibit the growth and production of verotoxins. In this study, acetic acid inhibited the in vitro growth of IPH9 four-fold stronger than lactic acid. The smaller dissociation constant of acetic acid may have caused the higher inhibition of IPH9, and the smaller molecule of acetic acid (60.05 daltons) should also facilitate rapid passage through the membrane (1). However, in the study of Buchanan and Edelson (6), lactic acid was more effective than acetic acid, but the authors stated that this result was caused by the acidic condition (pH 3.0). According to the literature, the growth of O157 seems to be more sensitive to acetic acid than lactic acid at higher pH values (4.7 to 6.0) (20, 22).

Compared with the results of cultivation in 0.1 mol l⁻¹ acetic acid-supplemented medium which showed the highest inhibition of O157 among various culture conditions, BB536-supernatant inhibited the growth of IPH9 to a lesser extent than acetic acid, but it showed a higher inhibition of extracellular verotoxin. In these culture experiments, the pH of all cultures after 24 hr was around 6.5. These data indicate that the inhibition of verotoxin by BB536 was not caused only by acids or a low pH. Some speculations can be made concerning inhibitory factors other than these two. It may be presumed that the reasons are as follows. One is the lack of nutrition (substrates for verotoxin production) in the BB536-supernatant, and the other is the presence of inhibitory factors in the BB536-metabolites. There are two similar reports on inhibitory effect of bifidobacterial supernatants. But one had no description of verotoxins (11), and in the other report, decrease of verotoxins seemed to be a result of O157 growth-inhibition (14). The inhibitory effect of BB536 may be remarkable for the production of verotoxin. Further studies are required to examine these inhibitions using different approaches.

In clinical treatment, destroying the O157 cell may incur the risk of increasing the spread of verotoxins to the intestine, and delicate management is necessary in administering antibiotics. The in vitro inhibitory effects of O157 exhibited by BB536 (particularly inhibition of verotoxin) observed in the present study may have important implications in O157-infection, and be attractive as probiotics for humans.

REFERENCES


