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Inhibition of enterohemorrhagic *Escherichia coli* O157:H7 infection in a gnotobiotic mouse model with pre-colonization by *Bacteroides* strains

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Abstract. Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 has been known to cause outbreaks of hemorrhagic colitis and hemolytic uremic syndrome. We previously demonstrated that intestinal flora contribute to the prevention of EHEC infection in a mouse model. However, it has not yet been determined whether *Bacteroides*, a predominant genus in the human intestine, contributes to the prevention of EHEC infection. The aim of the present study was to investigate the effect of *Bacteroides fragilis* (*B. fragilis*) and *Bacteroides vulgatus* (B. vulgatus) on EHEC O157:H7 infection *in vivo* using gnotobiotic mice. These strains were inoculated into germ-free mice to create a gnotobiotic mouse model. EHEC was inoculated into the mice, which were then monitored for 7 days for any change in symptoms. The mice that had been pre-colonized with the *Bacteroides* strains did not develop lethal EHEC infection, although several inflammatory symptoms were observed in the *B. vulgatus* pre-colonized group. However, no inflammatory symptoms were identified in the *B. fragilis* pre-colonized group. Moreover, *B. fragilis* exerted an inhibitory effect on enterocyte-like cell apoptosis. *B. fragilis* protected HT29 cells from apoptosis caused by Shiga toxin. In conclusion, the findings of the present study demonstrated that colonization by *Bacteroides* strains can inhibit EHEC infection.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is one of the most common pathogenic intestinal bacteria worldwide. EHEC is a food-borne zoonotic pathogen associated with outbreaks that pose a major public health concern worldwide. Once EHEC is ingested, it produces and releases Shiga toxin (Stx) (1). Stx is one of the most important pathogenic factors in EHEC infections (2). Stx binds to globotriaosylceramide (Gb3), which is a Stx receptor expressed in the intestinal epithelium and on the surface of endothelial cells (2). After Stx binds to Gb3, it inhibits protein synthesis and induces cell apoptosis (1). Gb3 is also expressed on vascular endothelial cells and nerve cells. Once Stx enters the bloodstream, it may lead to kidney and brain injury (3,4). Stx comprises Stx1 and Stx2 (1). Stx1 has the same structure as the Shiga toxin produced by *Shigella dysenteriae* (1), whereas Stx2 has a different structure (5), and it has been reported that Stx2 is associated with the severity of EHEC infection (5). EHEC colonizes the colon and causes diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS) or encephalopathy in humans (6,7). EHEC has several serotypes (8), and EHEC O157:H7 is the strain with the highest rate of isolation (1). In 1982, EHEC O157:H7 was isolated and identified in America as a food-borne pathogen (1). It was the first identification of a food-borne pathogen causing worldwide colitis outbreaks (9). In 1996, a big outbreak of EHEC O157:H7 infection occurred, starting with a school lunch in Japan (10). Therefore, EHEC O157:H7 has been recognized as one of the most serious food-borne pathogens.

In a previous study, it was reported that the susceptibility to EHEC infection varies among different individuals, with infants, children and the elderly being highly susceptible (11). In particular, patients younger than 5 years are at high risk for the development of severe symptoms, such as HUS (11).

Cattle are major carriers of EHEC; however, EHEC colonization in adult ruminants is asymptomatic (1). While EHEC colonizes the colon of humans and forms pathological lesions, it may colonize the recto-anal junction of cattle without Stx-related manifestations (1). The differential susceptibility to Stx and selectivity in colonization sites are associated with host tolerance to EHEC. Cattle transmit EHEC to humans by shedding the pathogen in the feces. Fecal shedding leads to contamination of farm environments by EHEC (12). In a recent study, Wang *et al* investigated the role of the microbiome in EHEC shedding, and indicated that shedding is

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affected by the composition of the microbiome (12). In particular, it was demonstrated that Firmicutes, Bacteroidetes and Proteobacteria promote EHEC shedding. These phyla also represent the predominant microorganisms in the human and mouse gut microbiome (13). Therefore, it was suggested that these strains may play an important role in EHEC infection in humans and mice.

Intestinal microbiota play an important role in protecting hosts from enteric infections. It has been reported that gastrointestinal microbiota act protectively against enteric infections (14-16). Furthermore, the susceptibility to EHEC infections is affected by the composition of the intestinal microbiome in mice (17). Several studies have investigated the association between specific bacterial strains and EHEC infection (18-23), focusing on probiotic strains. Probiotics are live organisms that, when ingested in adequate amounts, confer a health benefit on the host (24). By protecting the host from pathogen colonization (23) and modulating host immune response (25), probiotic bacteria can contribute to the defense against and recovery from pathogenic infections. In particular, *Bifidobacterium* and *Lactobacillus* strains are the predominant and subdominant groups of gastrointestinal microbiota, respectively (26). These strains are the most widely used probiotic bacteria and are included in a number of functional foods and dietary supplements (26-28). *Bifidobacterium* and *Lactobacillus* are highly relevant for the prevention of tissue invasion by enteropathogens (29).

It was recently reported that the ratio of *Bacteroides* in intestinal microbiota gradually increases with aging (30). *Bacteroides* is one of the most predominant microbial genera within the gastrointestinal tract (31). Furthermore, *Bacteroides* exerts negative effects on their hosts (31,32). It is generally considered that *Bacteroides* may promote infections and cause inflammatory diarrhea and ulcerative colitis, among others (33-35). However, to the best of our knowledge, the association between *Bacteroides* and EHEC infection has not yet been reported.

The aim of the present study was to examine the association between *Bacteroides* and EHEC infections. Two *Bacteroides* strains were used, namely *B. fragilis* and *B. vulgatus*. These strains generally promote infections (33-35). However, a recent study demonstrated that *B. fragilis* can modulate the host immune system (36), exerting not only negative but also positive effects on the host. Therefore, to elucidate the role of *Bacteroides* in intestinal microbiota, the association between *Bacteroides* and EHEC infection was investigated.

Materials and methods

Bacterial strains, media and cultures. EHEC O157:H7 EDL931k was obtained from the EDL931 strain (37). *B. fragilis* RIMD0230001 and *Bacteroides vulgatus* JCM5826 were the strains of *Bacteroides* used in the present study (36,38). EHEC and *Bacteroides* were propagated in 10 ml of brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI, USA) and Gifu Anaerobic Medium (GAM) broth (Nissui Pharmaceutical Co., Tokyo, Japan), respectively. All bacteria were incubated anaerobically in Anaero-Pack systems (Mitsubishi Gas Chemical, Tokyo, Japan) at 37˚C for 24 h. The BHI and GAM media were sterilized at 121˚C for 15 min and 115˚C for 15 min, respectively.

Table I. Definition of low, medium and high level of EHEC CFU, and Stx1 and Stx2.

Level	Number of EHEC (log_{10} CFU/ml)	Stx1 and Stx2
High	≥9.0	≥30.0
Medium	7.0-8.9	10.0-39.9
Low	≤6.9	≤9.9

EHEC, enterohemorrhagic *Escherichia coli*; CFU, colony-forming units; Stx, Shiga toxin.

Animals. Male germ-free (GF) mice (IQI/Jic, 5 weeks old) were obtained from Japan Clea Co. Ltd (Tokyo, Japan). Each group of mice was housed in a cage with a BBH box isolator on a 12:12 light:dark cycle at 24±2˚C under aseptic conditions. The mice were provided autoclaved diet and water *ad libitum*.

Cell culture. Enterocyte-like HT29 cells (39) were used for analysis with the MUSE Cell Analyzer (Merck KGaA, Darmstadt, Germany). HT29 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% sterilized fetal bovine serum (Valley Biomedical, Inc., Winchester, VA, USA) and 1% antibiotic/antimycotic mixed stock solution (Nacalai Tesque, Inc.). HT29 cells were incubated in 5% CO_2 at 37˚C. Cell treatment was performed as previously described (40).

Inoculation and EHEC infection. The EHEC infection protocols were based on the methods described by Isogai *et al* (41). The mice were divided into 6 groups as follows: *B. fragilis* pre-colonized group (with or without EHEC inoculation), *B. vulgatus* pre-colonized group (with or without EHEC inoculation), EHEC mono-colonized group and medium-only-inoculated mice. The EHEC mono-colonized group and medium-only-inoculated mice were used at the same time by Koyanagi *et al* (unpublished data).

The strains of *Bacteroides* were incubated overnight at 37˚C under anaerobic conditions and suspended at a concentration of 10^8 colony-forming units (CFU)/ml in sterile Dulbecco's phosphate-buffered saline (D-PBS) (Nissui Pharmaceutical). The suspension of *Bacteroides* strains (100 µl/mouse) was inoculated orally through a soft polyethylene catheter that was immediately removed. After 24 h of *Bacteroides* strain inoculation, 100 µl of the EHEC suspension ($1.0x10^7$ CFU/ml) or sterile BHI medium were inoculated in each mouse using the same method. Seven days after EHEC inoculation, the mice were sacrificed by cervical dislocation.

Histopathological analysis. The mouse kidneys and intestines were fixed overnight in 10% formaldehyde at ~25˚C and the

Table II. Effects of bacterial colonization on mouse lethality 7 days after EHEC O157:H7 infection.

Groups	EHEC inoculation	Total no. of mice	No. of mice	
			Dead	Exhibiting intestinal edema and hemorrhagic lesions
B. fragilis pre-colonized group	+	5	0[a]	0[a]
	-	5	0	0
B. vulgatus pre-colonized group	+	4	1	4
	-	4	0	0
EHEC mono-colonized group	+	4	4	4
Medium-only-inoculated mice	-	3	0	0

[a]Significant differences compared with EHEC mono-colonized group were showed using Steel's test ($P<0.05$). EHEC, enterohemorrhagic *Escherichia coli*.

tissues were embedded in paraffin and stained with hematoxylin and eosin.

Confirmation of EHEC translocation to organs. Seven days after EHEC inoculation, the mice were dissected and the lungs, liver, spleen, brain and heart were removed. The sections of these organs were stamped on CHROMagar™ O157 for the detection of EHEC O157:H7 (CHROMagar Microbiology, Paris, France). After incubation for 48 h at 37˚C under aerobic conditions, colony formation was examined.

EHEC count and Stx detection in mouse fecal samples. At 1, 3 and 7 days after the inoculation of EHEC, feces were collected from mice in different groups. Fecal samples were suspended in BHI broth at a 1:19 (w/v) ratio. To quantify the number of colonized EHEC, fecal suspensions were serially diluted and plated on CHROMagar™ O157 for detection of EHEC O157:H7. After 48 h of anaerobic incubation at 37˚C, the CFU/ml of EHEC O157:H7 was determined. Stx1 and Stx2 titers were qualified using a verotoxin detection kit based on reserved passive latex agglutination (Denka Seikan Co., Ltd., Tokyo, Japan). The fecal suspensions were centrifuged at 900 x g for 10 min at room temperature, and the supernatant was used for Stx1 and Stx2 detection. The number of EHEC and the levels of Stx1 and Stx2 we defined as low, medium and high. The ranges are defined and provided in Table I.

Suppression of apoptosis caused by Stx using Annexin V and 7-AAD combination assays. Muse Annexin V and Dead Cell kit (Merck KGaA) was used for the detection of apoptosis in this experiment. This kit has been used previously for sensitive detection of apoptosis (42,43). HT29 cells were seeded at a density of $1.0x10^5$ cells/well in a 12-well plate and incubated at 37˚C and 5% CO_2 until reaching confluence. The DMEM was replaced with 900 μl fresh medium without antibiotic/antimycotic mixed stock solution 30 min prior to bacterial inoculation. Culture solutions of *Bacteroides* strains incubated in GAM broth overnight were adjusted to $1.5x10^8$ CFU/ml and resuspended in PBS. Following incubation for 30 min in DMEM without antibiotic/antimycotic mixed stock solution, 1 ml *Bacteroides* suspension was inoculated into the cells. Similarly, culture solutions of EHEC incubated in BHI broth overnight were adjusted to $1.5x10^8$ CFU/ml and resuspended in PBS. After 1 h of incubation, 100 μl EHEC suspension was inoculated into the cells. After 9 h, the culture supernatants were collected for Stx detection, as described above. Subsequently, the cells were washed 3 times with 1 ml PBS, treated with trypsin and transferred into microtubes. The cells were centrifuged at 800 x g for 5 min and resuspended in 100 μl fresh DMEM. A total of 100 μl Annexin V and Dead Cell Dye assay reagent (Merck KGaA) were added to the samples and mixed. After incubation for 20 min at room temperature in the dark, the samples were applied to the Muse Cell Analyzer (Merck KGaA).

Statistical analysis. Significant differences in lethality and EHEC translocation were calculated using the Steel's test. Furthermore, the statistical differences in EHEC viable counts, Stx levels in fecal samples and apoptotic cells in the co-culture of *Bacteroides* and EHEC were determined by Dunnett's test or the Tukey-Kramer test. Significant differences were defined as probability values of <0.05. Experiments *in vitro* were performed in triplicates or more.

Results

Prevention of EHEC infection-related lethality by Bacteroides colonization. The effect of intestinal *Bacteroides* strains against EHEC infection was examined using GF mice. Colonization with *B. fragilis* was found to significantly decrease the lethality of EHEC infection ($P<0.05$; Table II). In the *B. fragilis* pre-colonized group, all mice survived until day 7, whereas in the *B. vulgatus* pre-colonized group, 25% of the mice died within the first 5 days while the rest survived until day 7. However, all the mice in the EHEC-mono-colonized group had died by day 5. All the mice of the EHEC-mono-colonized and *B. vulgatus* pre-colonized groups exhibited intestinal edema and hemorrhagic lesions (Table II).

Suppressive effects of colonization by Bacteroides strains on the histopathological changes in the intestine and kidney. As determined by histological analysis, *B. fragilis* protected

Table III. Effects of bacterial colonization on EHEC translocation to organs.

Groups	EHEC inoculation	Total no. of mice	No. of mice detected with EHEC in each organ			
			Heart	Liver	Spleen	Kidney
B. fragilis-colonized group	+	5	0	0	0	0
	-	5	0	0	0	0
B. vulgatus-colonized group	+	4	1	2	3	1
	-	4	0	0	0	0
EHEC-infected GF mice	+	4	2	3	3	3
Medium-only-inoculated mice	-	3	0	0	0	0

EHEC, enterohemorrhagic *Escherichia coli*; GF, germ-free.

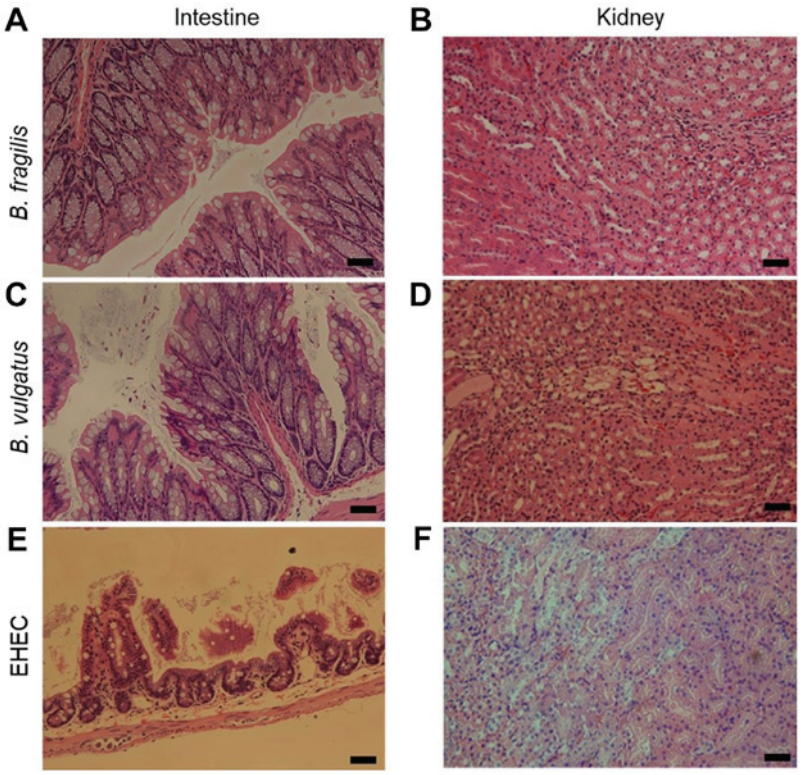

Figure 1. Histopathological changes in the intestine and kidney. Hematoxylin and eosin staining of the (A, C and E) intestine and (B, D and F) kidney in mice infected with EHEC. Panels A and B, *B. fragilis*-colonized mouse almost fully suppressed the inflammatory symptoms. Panels C and D, *B. vulgatus*-colonized mouse exhibited neutrophil migration in the intestine and cytopathic alterations in the kidneys. Panels E and F, EHEC-mono-colonized mouse showed shedding of epithelial cells in the intestine and necrosis of renal tubules in the kidney. Bars, 50.0 μm. EHEC, enterohemorrhagic *Escherichia coli*.

the host from the development of histopathological lesions and almost fully suppressed the inflammatory symptoms caused by EHEC inoculation (Fig. 1A and B). *B. vulgatus* also protected the host from death following EHEC inoculation (Table II); however, neutrophil migration was observed in the intestine and cytopathic changes were observed in the kidneys (Fig. 1C and D). In the EHEC mono-colonized group, shedding of epithelial cells was observed in the intestine, and necrosis of renal tubules was observed in the kidney (Fig. 1E and F).

Suppression of EHEC translocation to other organs by Bacteroides colonization. In the *B. fragilis* pre-colonized group, no translocation of EHEC was observed (Table III). However, in the *B. vulgatus* pre-colonized and EHEC-mono-colonized groups, EHEC translocation to other organs, such as the heart, liver, spleen and kidney, was observed. When comparing the translocation rate between *B. vulgatus* and the EHEC group, translocation in the EHEC mono-colonized group was higher compared with that in the *B. vulgatus* pre-colonized group, but the difference between the two groups was not statistically significant (P=0.13).

Comparison of the effects of Bacteroides colonization on viable counts of EHEC O157:H7. On day 1, the EHEC viable count in

Table IV. Excretion levels of EHEC, Stx1 and Stx2 in the feces.

Groups	EHEC inoculation	Total no. of mice	EHEC colonization level	Stx level in the feces			
				Stx 1		Stx 2	
			Day1[a]	Day 3	Day 7	Day 3	Day 7
B. fragilis-colonized group	+	5	L	M[b]	L	M[b]	L
	-	5	ND	ND	ND	ND	ND
B. vulgatus-colonized group	+	4	M	M[b]	M	H[b]	M
	-	4	ND	ND	ND	ND	ND
EHEC-colonized group	+	4	H	H[c]	NT	H[c]	NT
Medium-only-inoculated group	-	3	ND	ND	ND	ND	ND

[a]Data of days 3 and 7 not shown as no significant differences were observed among groups. Stx levels are shown as final dilution ± standard deviation. H, high; M, medium and L, low (Table I). EHEC, enterohemorrhagic *Escherichia coli*; Stx, Shiga toxin; ND, not detected; NT, not tested due to death. [b,c]No significant difference at the 99% confidence level, using the Tukey-Kramer test.

the *B. fragilis* pre-colonized group was lower compared with that in the EHEC mono-inoculated group (P=0.0538), although the difference was not significant (Table IV). However, the EHEC count gradually increased from day 3 to day 7. In the *B. vulgatus* pre-colonized group, no significant differences were observed among the different time points. Furthermore, the EHEC count in the EHEC-mono-colonized group was not examined on day 7, as all the mice had died by day 5 following EHEC inoculation.

Inhibitory effects of colonization by Bacteroides strains on Stx1 and Stx2 levels in fecal samples. In the *B. fragilis* pre-colonized group, Stx1 and Stx2 levels were significantly lower compared with those in the EHEC mono-colonized group on day 3 (Table IV). Furthermore, in the *B. vulgatus* pre-colonized group, the Stx1 level was significantly lower compared with that in the EHEC group. By contrast, no significant differences were observed in Stx2 levels between the *B. vulgatus* pre-colonized and the EHEC-mono-colonized groups on day 3. EHEC mono-colonized mice exhibited >10-fold higher Stx2 levels compared with mice colonized with *Bacteroides*. The Stx levels in the EHEC mono-colonized group were not tested on day 7, as all the mice had died by day 5 following EHEC inoculation.

Detection of apoptosis of HT29 cells co-cultured with Bacteroides strains and EHEC. The apoptosis of epithelial cells was detected to investigate the factors mediating the protective effects of *Bacteroides* strains *in vitro*. EHEC O157:H7 is generally known to promote apoptosis of intestinal epithelial cells (1,44). However, in this experiment, in the *B. fragilis*-colonized group, no tissue lesions were observed in the small intestine (Fig. 1A), suggesting that *B. fragilis* exerted inhibitory effects on the apoptosis of epithelial cells. Therefore, the inhibitory effect of apoptosis was further examined in the *B. fragilis* strain.

In the *B. fragilis* and *B. vulgatus* mono-colonized groups, the majority of cells were non-apoptotic (Fig. 2A and B). However, in the EHEC-mono-colonized group, most cells were apoptotic or necrotic. Mono-colonization by EHEC was significantly increased during early apoptosis (Fig. 2C; P<0.01). Interestingly, co-culture with *B. fragilis* and EHEC significantly decreased the apoptotic cell percentage (P<0.01). However, co-culture with *B. vulgatus* and EHEC did not significantly affect apoptosis. Furthermore, Stx1 and Stx2 production by EHEC was not significantly suppressed in cells co-cultured with *B. fragilis* or *B. vulgatus* (Fig. 3).

Discussion

It has been reported that *B. fragilis* contributes to diarrheal disease in animals and humans (31), and that *B. vulgatus* is pathogenic in individuals with underlying conditions, such as patients with ulcerative colitis (45). In the present study, we demonstrated the protective effects of *Bacteroides* against EHEC infection. The findings of the study revealed that intestinal flora are implicated in the susceptibility to EHEC infection. In fact, GF mice inoculated with EHEC displayed severe symptoms and high lethality (Fig. 1, Table I). By contrast, colonization by a single *Bacteroides* strain exerted a protective effect. *B. fragilis* suppressed lethality from EHEC infection. Similarly, *B. vulgatus* suppressed EHEC lethality, albeit to a lesser extent. Furthermore, the EHEC count in the intestines of mice colonized by *B. fragilis* or *B. vulgatus* was reduced, although the difference was not significant (Table IV). Moreover, Stx production in the mouse intestine was significantly suppressed in the *B. fragilis*-colonized group (Table IV). These results demonstrated that *B. fragilis* and *B. vulgatus* effectively decreased the lethality of EHEC infection (Table II), particularly in the *B. fragilis* pre-colonized group (P<0.05). In the present study, the mechanisms by which each bacterium protected mice from EHEC infection were not fully elucidated. However, to the best of our knowledge, this study is the first to demonstrate that *Bacteroides* strains may act protectively against lethal EHEC infection in mice.

Our study suggested that the EHEC count in the early stages of EHEC infection is a key factor affecting the severity of the infection. Frankel *et al* reported that the locus of enterocyte effacement type III secretion system of EHEC is crucial for bacterial adhesion to the host's intestinal cells during the

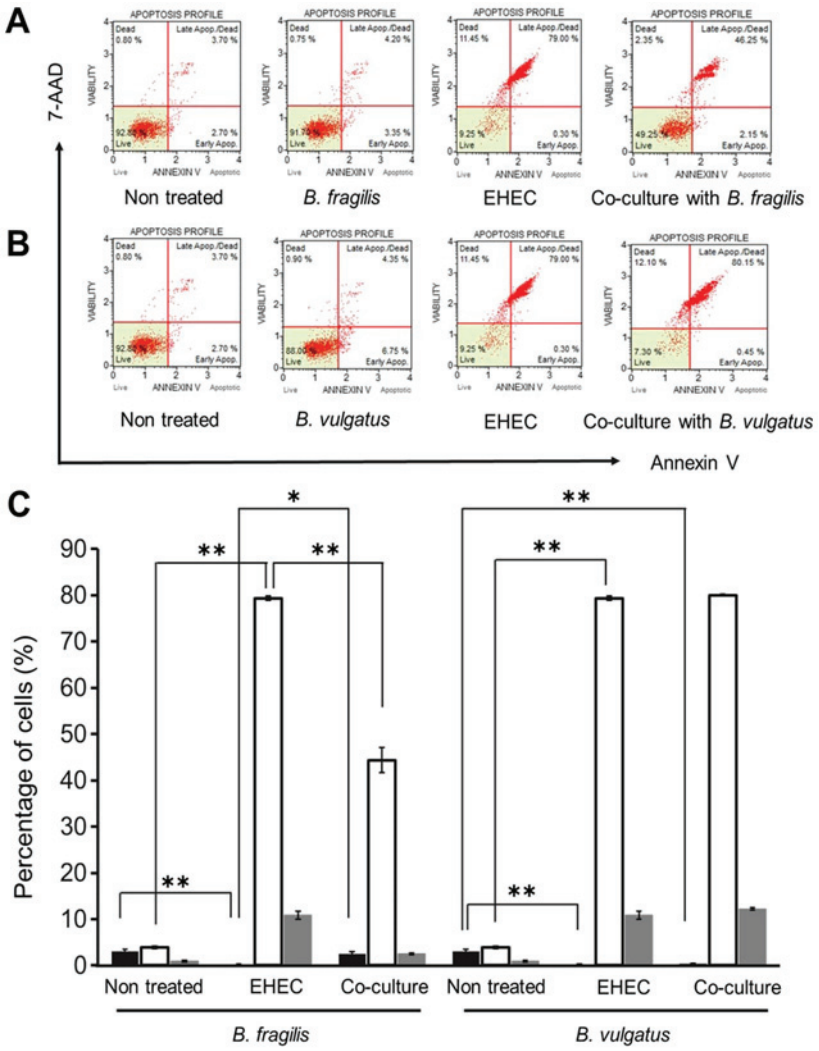

Figure 2. Detection of apoptosis in the EHEC mono-colonized and EHEC co-cultured with *Bacteroides* strains groups using flow cytometric analysis of apoptotic cells (n=3). (A and B) Results in non-treated HT29 cells, HT29 cells infected with EHEC, HT29 cells cultured with *Bacteroides* strains (A; *B. fragilis*, B; *B. vulgatus*), and HT29 cells infected with EHEC and prophylactically co-cultured with *Bacteroides* strains (A; *B. fragilis*, B; *B. vulgatus*). (C) Percentage of early apoptotic cells (black bar), late apoptotic or necrotic cells (white bar) and necrotic cells (gray bar). **P<0.01 and *P<0.05, statistically significant as calculated by the Tukey-Kramer test. Data are shown as mean ± standard deviation of three different experiments. EHEC, enterohemorrhagic *Escherichia coli*.

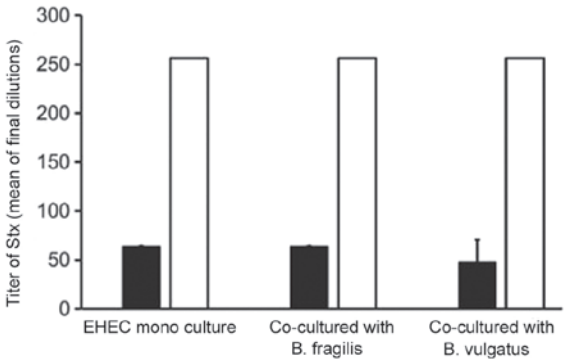

Figure 3. Quantification of Stx1 (black bar) and Stx2 (white bar) levels in the EHEC mono-colonized and EHEC co-cultured with *Bacteroides* strains groups (n=3). Data are shown as mean ± standard deviation of three different experiments. The Tukey-Kramer test revealed no statistically significant differences. EHEC, enterohemorrhagic *Escherichia coli*.

early stages of infection (46). Adhesion to the epithelial cells enables disease establishment (47). The results of our study demonstrated that *B. fragilis* lowered the EHEC count *in vivo* (P=0.0538; Table IV). In addition, *B. fragilis* fully protected the host from EHEC infection and suppressed the formation of pathological lesions (Fig. 1A and B; Table II). However, *B. vulgatus* did not lower the EHEC count in the early stages of infection (Table IV) and did not completely suppress the development of symptoms (Table II). Therefore, the type of *Bacteroides* colonization and the early stages of the EHEC infection are key factors in determining disease severity.

The present study also demonstrated that translocation may be another factor associated with the severity of EHEC infection. Generally, EHEC is taken up orally and colonizes the intestinal tract (1). Considering the route of EHEC translocation, if the barrier of the intestinal tract wall is compromised, Stx can circulate in the entire body. Fukuda *et al* reported that Stx translocation is associated with EHEC infection lethality (23). In the present study, the *B. fragilis*-colonized group did not exhibit EHEC translocation to the other organs examined (Table III), and there were no pathological lesions identified (Fig. 1). However, in the *B. vulgatus* pre-colonized

and EHEC-mono-colonized groups, EHEC translocation was observed in all the organs examined (Table III). In addition, the EHEC-mono-colonized group displayed the highest ratio of EHEC translocation in each organ (Table III). Furthermore, Stx levels in the feces were examined and the *B. fragilis* pre-colonized group exhibited the lowest Stx level among all groups (Table IV). Therefore, the findings of the present study demonstrated that *B. fragilis* suppressed the susceptibility to EHEC infection.

Protecting the intestinal tract helps prevent lethal EHEC infections (16). Stx produced by EHEC promotes apoptosis of intestinal epithelial cells (23). Inhibition of Stx circulation in the body is crucial for the prevention of lethal EHEC infection (16). In the present study, the inhibitory effect of *Bacteroides* on epithelial intestinal cell apoptosis was demonstrated (Fig. 2). In the EHEC-mono-colonized group, the majority of the cells were apoptotic *in vitro* (Fig. 2C). However, in the *B. fragilis*-co-cultured group, apoptosis of HT29 cells was significantly reduced ($P<0.01$; Fig. 2C), whereas apoptosis was not suppressed in the *B. vulgatus*-co-culture group (Fig. 2C). Of note, Stx production was not found to be significantly suppressed following apoptosis analysis (Fig. 3). The reason apoptosis was suppressed in HT29 cells remains unclear, and the underlying mechanisms were not elucidated in the present study. However, *B. fragilis* was confirmed to exert an inhibitory effect on intestinal epithelial cell apoptosis.

In conclusion, the present study demonstrated that *Bacteroides* prevented EHEC infection. It was also suggested that *Bacteroides* may be associated with susceptibility to EHEC infection in mice, in addition to cattle. Our findings using single-flora systems demonstrated that *Bacteroides* contributed to the prevention of EHEC infection, and *B. fragilis* was shown to fully protect against EHEC infection. The interaction between EHEC and *Bacteroides* in GF mice provides little information regarding their behavior in the microbiome. However, understanding the role of each intestinal bacterium is relevant when considering treatment against EHEC infection. Further studies are required to elucidate the mechanism underlying the protective role of *B. fragilis* against EHEC infection.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Ethics approval and consent to participate

Each experimental protocol was performed in accordance with the Regulations for Animal Experiments and Related Activities at Tohoku University (approval no. 2011AgA-30).

Patient consent for publication

Not applicable.

Authors' contributions

KS, RS, HI and EI performed the experiments; KS, RS, YK and EI designed the study; KS, YK, HY and EI wrote the manuscript.

Competing interests

The authors declare that they have no competing interests to disclose.

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