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Original Article

The effect of gut microbiota and probiotic organisms on the properties of extended spectrum beta-lactamase producing and carbapenem resistant *Enterobacteriaceae* including growth, beta-lactamase activity and gene transmissibility☆,☆☆

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ABSTRACT

The gut microbiota may play a pivotal role in controlling the antimicrobial resistant (AMR) organisms although the evidences are limited. We investigated the effects of gut microbiota on the growth of AMR organisms, β-lactamases activity and transmissibility of antimicrobial resistant properties of the extended spectrum β-lactamase (ESBL)-producing *Escherichia coli* and carbapenem-resistant *Enterobacteriaceae*.

CTX-M-15-positive, ESBL-producing *E. coli* and carbapenem resistant *Enterobacteriaceae*, *Bacteroides fragilis*, *Bifidobacterium longum*, *Clostridium butyricum*, *Clostridioides difficile*, *Clostridium perfringens*, *Enterococcus faecium*, *Lactobacillus plantarum* and probiotic strain of *C. butyricum* MIYAIRI 588 were used in this study.

The growth of AMR organisms was suppressed by the supernatant of *C. butyricum*, *C. difficile*, *C. perfringens*, *E. faecium* and *L. plantarum* in a dose dependent manner but not by that of *B. fragilis* and *B. longum*. The β-lactamase activity produced by *E. coli* was reduced by the presence of culture supernatant of certain gut microbiota during stationary phase of *E. coli*. Importantly, *C. butyricum* MIYAIRI 588 culture supernatant suppressed the transcription of bla_{CTX-M} gene during growth phase of *E. coli*. The conjugation assay showed the reduction of transmissibility of antibiotic resistant gene by gut microbiota.

These findings suggest that certain gut microbiota affect the antibiotic resistant activities of AMR organisms. Further studies are needed to identify the specific mechanism(s) of these actions between AMR organisms and gut microbiota.

1. Introduction

The emergence of antimicrobial resistant (AMR) bacteria has become a worldwide concern. Efforts have been made to overcome the problem by accelerated antibiotic development with new mode of action. However, even if we were to develop a new generation of antibiotics, bacteria will rapidly acquire the resistance to the newly developed antibiotics and prevail as multidrug resistant strains.

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Therefore, a novel approach to control the AMR bacteria is inevitable for avoiding future problems.

The extended spectrum β-lactamase (ESBL) is a group of β-lactamase enzymes that acquired the extended ability to inactivate new β-lactam antibiotics such as third generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime) as well as monobactams produced by *Klebsiella pneumoniae* and *Escherichia coli* known to spread worldwide since 1980s [1,2]. Paterson and Bonomo [3] reported that many peer-reviewed medical articles and case controlled studies have shown the risk of nosocomial infection with ESBL-producing organisms.

CTX-M type ESBLs are the most widespread since the $bla_{CTX\text{-}M}$ genes located on plasmids are readily transmissible by conjugation. Therefore, the ESBL producing ability can be easily acquired from $bla_{CTX\text{-}M}$ genes positive organisms to the other *Enterobacteriaceae* [4,5]. It is well known that the endogenous microbiota in digestive tract is a reservoir of AMR organisms, serving as a melting pot where exchanges of AMR genes occur. Especially when antibiotics are used extensively, it selects for the emergence of AMR bacteria [6]. However, little is known about the direct relationship between gut microbiota and AMR organisms such as activities of the horizontal transmissibility of the AMR genes. Therefore, in this study we examined the potential properties of gut microbiota on the growth of AMR organisms and the transmissibility of AMR genes.

The clinical effects of probiotics have been documented on irritable bowel syndrome, inflammatory bowel diseases as well as several infectious diseases [7]. Particularly, the effects of probiotics have been well demonstrated on the antibiotic associated diarrhea (AAD) due to *Clostridioides difficile* infection and peudomembranous colitis (PMC) [8,9] where probiotics are often co-prescribed with antibiotics to alleviate such conditions.

The multi factorial mechanism of actions by probiotics has been implicated to reduce the risk against infectious diseases; However, few scientific studies have been done on the inhibitory effects of probiotics on ESBL gene expression, β-lactamases activity and/or transmissibility of AMR genes.

Clostridium butyricum is a Gram positive, spore forming obligate anaerobe found in soil and intestine, of which MIYAIRI 588 strain is one of probiotics well prescribed to prevent AAD and other gastrointestinal diseases [10]. The mechanisms of protective effects by *C. butyricum* MIYAIRI 588 strain against the infection by several pathogens have been reported including the production of antagonistic substances which inhibit or kill the pathogen [11,12], the competition with the pathogens for adhesion to enterocyte [11] and the inhibition of toxin production at phenotypic and molecular level [13]. These studies suggest that *C. butyricum* MIYAIRI 588 strain may produce an active molecule(s) which results in prevention and/or treatment of AAD in the clinical practice [14]. Furthermore, if probiotics including *C. butyricum* MIYAIRI 588 strain can suppress the growth of ESBL-producing organisms or reduce the activity of β-lactamases, this may improve clinical outcomes when treating such resistant infections.

Therefore, this *in vitro* study was designed to investigate the correlation between gut microbiota and ESBL-producing microorganisms in terms of growth, β-lactamase activity and transmissibility of the AMR genes from $bla_{CTX\text{-}M}$ positive, ESBL-producing *E. coli* and carbapenem resistant enterobacteriaceae, the most emerging AMR organisms.

2. Materials and methods

2.1. Bacterial strains

Total 8 clinical isolates of ESBL-producing *E. coli* or carbapenem resistant *Enterobacteriaceae* (CRE) were used as a resistant strain (Table 1) [15]. All strains were rifampicin-sensitive and β-galactosidase-positive. Our laboratory stocked type strain of *Bacteroides fragilis* ATCC 25285^T, *Bifidobacterium longum* JCM 1217^T, *C. butyricum* ATCC 19398^T, *C. difficile* ATCC 9689^T, *Clostridium perfringens* JCM 1290^T, *Enterococcus faecium* JCM 5804^T and *Lactobacillus plantarum* ATCC 1149^T were used as a clinically important or representative of gut microbiota. *C. butyricum* MIYAIRI 588 was used as a probiotic strain. A laboratory strain of *E. coli* TUM 5480 derived from K-12 (rifampicin-resistant, cefotaxime-sensitive and β-galactosidase-negative) was also used as the recipient in the conjugation assay that was kindly provided by Professor Yoshikazu Ishii of the Department of Microbiology and Infectious Diseases, Toho University School of Medicine.

2.2. Preparation of culture supernatant

Single colony of *B. fragilis*, *B. longum*, *C. butyricum*, *C. difficile*, *C. perfringens*, *E. faecium* and *L. plantarum*, which was separately grown on BL agar (Nissui pharmaceutical, Tokyo, Japan) supplemented with 5% defibrinated horse blood, was inoculated into BHI broth (Becton Dickinson, NJ, USA) and incubated at 37 °C for 24 h under the anaerobic condition (10% H_2, 10% CO_2 and 80% N_2) using an anaerobic chamber. After the neutralization into pH7.0 by using 1 N NaOH, the culture was centrifuged at 5000×*g* for 10 min and filtered using 0.45 μm pore-size filter.

2.3. Growth and β-lactamase inhibition assay

The growth of ESBL-producing *E. coli* or CRE was examined in BHI broth under the presence of culture supernatant of gut microbiota or probiotic strain at final concentrations of 0% (negative control, without culture supernatant), 50% (diluted by BHI broth medium) and 100% culture supernatant. Overnight culture of resistant strain was inoculated at initial number of ca. 5×10^3 cfu/ml in the medium supplemented with or without culture

Table 1
MICs and Types of *bla* gene in ESBL producing *E. coli* and CRE in this study.

Strain	MIC	Types of *bla* gene					
		CTX-M	IMP-1	IMP-2	VIM-2	OXA-48	KPC
E. coli Tohoku 9089	CAZ, CTX ≥256	+	NT	NT	NT	NT	NT
K. pneumoniae strain no.1	MEPM = 4	+	+	−	−	−	−
K. pneumoniae strain no.2	MEPM = 4	+	+	−	−	−	−
K. pneumoniae strain no.3	MEPM = 16	+	+	−	−	−	−
K. pneumoniae strain no.4	MEPM = 8	+	+	−	−	−	−
K. pneumoniae strain no.5	MEPM = 8	+	+	−	−	−	−
K. pneumoniae strain no.6	MEPM = 16	−	+	−	−	−	−
E. coli strain no.1	MEPM = 4	−	+	−	−	−	−

CAZ: ceftadizime; CTX: cefotaxime; MEPM: meropenem; NT: Not tested.

supernatant and incubated anaerobically at 37 °C. Culture of resistant strain was sampled with time, the number of live bacteria and β-lactamase activity were examined. Modified Drigalski agar (Becton Dickinson, NJ, USA) was used for enumeration for resistant bacteria.

2.4. Determination of β-lactamase activity

The β-lactamase activity was determined by using the chromogenic substrate nitrocefin according to the description by others [16,17]. Culture of resistant bacteria was centrifuged at 5,000×*g* for 10 min and washed twice with equal volume of 50 mM phosphate buffer (pH 7.0) (PB) and re-suspended in 1/10 volume of PB. The cell suspension was sonicated for 3min on ice and centrifuged at 13,000×*g* at 4 °C for 30min, and the obtained supernatant was used as a crude enzyme solution. The protein concentration in samples was measured by Lowry method [18] using a bovine serum albumin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as a standard. 70 μl of samples diluted with PB at the protein concentration of 100 μg/ml was added into mixture of 35 μl of 1 mM nitrocefin solution (OXOID, Basingstoke, UK) and 595 μl of PB, the rate of nitrocefin hydrolysis was measured at 390 nm at 30 °C for 2min using a spectrophotometer (UV-2550, Shimadzu Corporation, Kyoto, Japan). One unit of β-lactamase activities are presented as 1 μmol of nitrocefin hydrolyzed per min per mg of protein.

2.5. Determination of gene expression of $bla_{CTX\text{-}M}$

Gene expression of $bla_{CTX\text{-}M}$ was analyzed by two-step quantitative reverse transcription-PCR by using the samples collected during the analysis of β-lactamase activity. Total RNA was isolated using a modified acid guanidinium thiocyanate-phenol-chloroform extraction method [19]. Briefly, a sample was resuspended in a solution containing 346.5 μl lysis buffer (QIAGEN, Hilden, Germany), 3.5 μl β-mercaptoethanol (Kanto Chemical Inc., Tokyo, Japan) and 100 μl of Tris -EDTA buffer (pH 8.0). Then 300 mg of glass beads (diameter 0.1 mm) was added to the suspension, and the mixture was vortexed vigorously for 20s using a MagNa Lyzer (Roche, Mannhein, Germany) at a speed level of 7,000 rmp. Then 500 μl acid phenol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added, the mixture was incubated for 10 min at 60 °C. After incubation, the mixture was cooled on ice for 5min and added to 100 μl chloroform-isoamyl alcohol (24: 1). After centrifugation at 12,000×*g* for 10 min at 4 °C, supernatant was collected and add to an equal volume of chloroform-isoamyl alcohol (24: 1). After centrifugation at 12,000×*g* for 10 min at 4 °C, supernatant was collected and subjected to isopropanol precipitation and washed with 70% ethanol. Finally, the nucleic acid fraction was suspended in 50 μl nuclease-free water. To quantity of RNA was confirmed spectrophotometrically using K2800 Nucleic Acid Analyzer (Beijing Kaiao Technology Department, Beijing, China), then total RNA was adjusted 400 ng/μl. Reverse transcription was conducted on extracted RNA using PrimeScript RT Master Mix (TaKaRa Bio Inc., Shiga). Real-time PCR of the c-DNA was performed using two sets of primer targeting the $bla_{CTX\text{-}M}$ (F: 5′-CGCTTTGCGATGTGCAG-3′, R: 5′-ACCGCGATATCGTTGGT-3′) [20] and the housekeeping *gapA* gene (F: 5′-GATTACATGGCATACATGCTG-3′, R: 5′-CAGACGAACGGTCAGGTCAA-3′) [21], and SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Bio Inc., Shiga). The amplification program consisted of one cycle at 95 °C for 30s, followed by 40 cycles at 95 °C for 5s and 60 °C for 1min. A melting curve analysis was performed after amplification to distinguish the target from the nontargeted PCR products. Amplification and detection were performed with a Thermal Cycler Dice Real Time System TP900 (TaKaRa Bio Inc., Shiga). The slope of calibration curve was used to compare the number of copies of each gene in 1 ml medium of *E. coli* 9089 strain at 24 h on control.

2.6. Conjugation assay

A conjugation assay was performed by the broth mating method [22] to determine the effect of gut microorganism or probiotic strain on the transmissibility of antibiotic resistance properties. Briefly, ESBL-producing *E. coli* or CRE as the donor strain and *E. coli* TUM 5480 as the recipient strain were co-cultured for 2 h at 37 °C under anaerobic conditions with or without supplementation of culture supernatant of gut microorganism or probiotic strain. After broth mating, transconjugants were selected by growth on Modified Drigalski agar (Becton Dickinson, NJ, USA) supplemented with appropriate concentrations of rifampicin and cefotaxime or meropenem. The frequency of transfer was then expressed relative to the number of donor cells or recipient cells.

3. Results

3.1. Effects of gut microbiota and probiotic strain on the growth of resistant bacteria

The effect of culture supernatant of gut microbiota and probiotic *C. butyricum* MIYAIRI 588 strain on the growth of ESBL producing *E. coli* was shown in Fig. 1. The growth of ESBL-producing *E. coli* Tohoku 9089 was suppressed by the supernatant of *C. butyricum*, *C. difficile*, *C. perfringens*, *E. faecium* and *L. plantarum* in a dose dependent manner. The inhibitory effects of *C. butyricum* ATCC 19398 and MIYAIRI 588 strain were observed through the whole growth stage of *E. coli*. On the other hands, the inhibitory effects of *C. difficile* was limited at exponential growth phase of *E. coli*, and those of *C. perfringens*, *E. faecium* and *L. plantarum* were limited at stationary phase. *B. fragilis* and *B. longum* had no inhibitory effects on the growth of *E. coli*. The inhibitory effects of culture supernatant of *C. butyricum* MIYAIRI 588 strain on the growth of various CREs were also observed similar as ESBL-producing *E. coli* Tohoku 9089 (Fig. 2).

3.2. Effect of gut microbiota and probiotic strain on the activity of β-lactamase

In addition to growth suppression, the activity of β-lactamase from ESBL-producing *E. coli* was also examined (Fig. 1). All gut microbiota and *C. butyricum* MIYAIRI 588 supernatant significantly reduced the β-lactamase activities at 24 h. The inhibitory effects of *B. fragilis*, *B. longum*, *C. butyricum* ATCC19398, *C. difficile*, *C. perfringens*, *E. faecium* and *L. plantarum* were not observed at 6 or 9 h. In contrast to that, *C. butyricum* MIYAIRI 588 showed inhibitory effects at all examined timing as of 6, 9 and 24 h and those activities were relatively strong compared to those of gut microbiota. At the time of 9 h cultivation of *E. coli*, the promoting effect of *L. plantarum* on the activity of β-lactamase was observed. *C. butyricum* MIYAIRI 588 also showed similar effects on the activity of β-lactamase from various CREs. (Fig. 2).

3.3. Inhibitory effects of C. butyricum MIYAIRI 588 on the expressions of $bla_{CTX\text{-}M}$ gene in ESBL-producing E. coli Tohoku 9089

The expression levels of $bla_{CTX\text{-}M}$ gene in ESBL-producing *E. coli* Tohoku 9089 under the presence of *C. butyricum* MIYAIRI 588 supernatant were examined (Fig. 3). *C. butyricum* MIYAIRI 588 showed inhibitory effects on the expression of $bla_{CTX\text{-}M}$ gene in dose dependent manner.

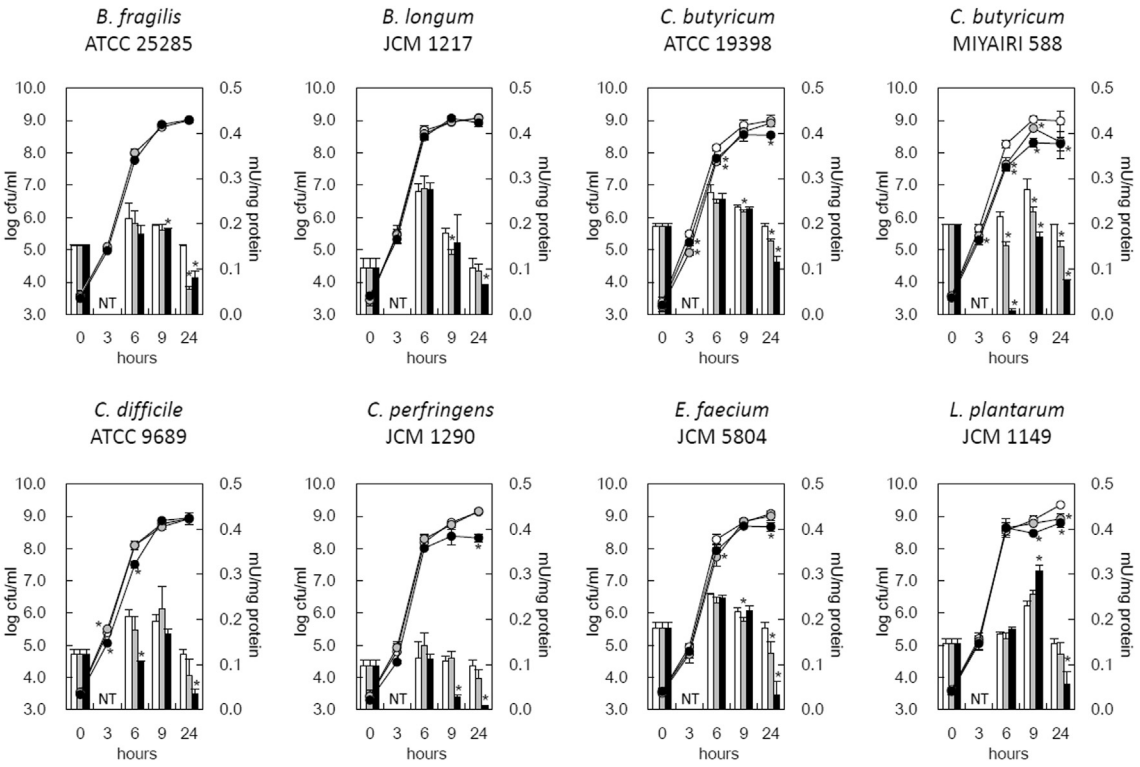

Fig. 1. Effects of culture supernatant of gut microbiota on growth and β-lactamase activity of ESBL-producing *E. coli* Tohoku 9089. Circle and line: growth of bacteria; Bar: β-lactamase activity. White: control (culture supernatant 0%); Gray (culture supernatant 50%); Black (culture supernatant 100%). Data were shown as mean ± SD. *: $p < 0.05$ vs Control. NT: Not tested.

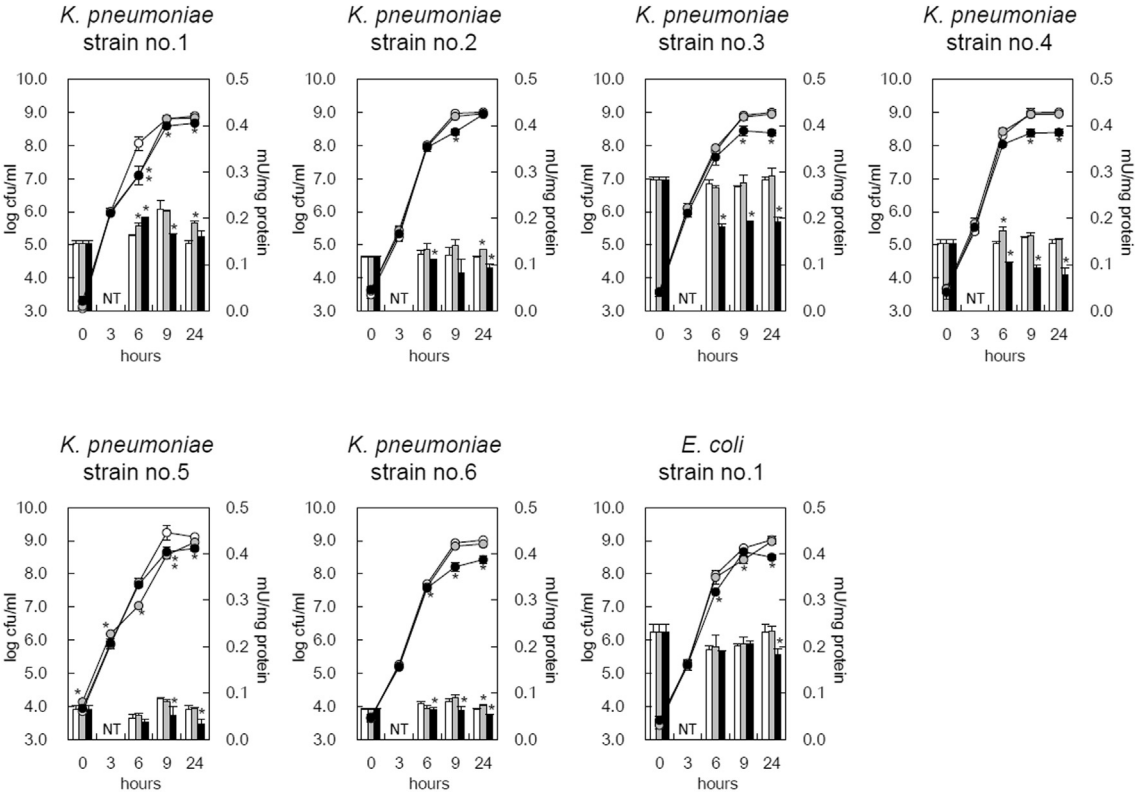

Fig. 2. Effects of culture supernatant of *C. butyricum* MIYAIRI 588 on growth and β-lactamase activity of CREs. Circle and line: growth of bacteria; Bar: β-lactamase activity. White: control (culture supernatant 0%); Gray (culture supernatant 50%); Black (culture supernatant 100%). Data were shown as mean ± SD. *: $p < 0.05$ vs Control. NT: Not tested.

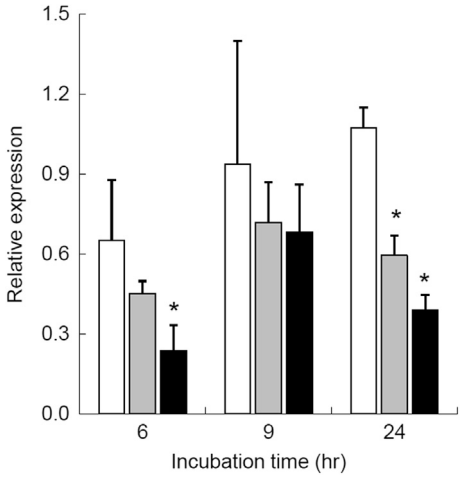

Fig. 3. Effects of culture supernatant of *C. butyricum* MIYAIRI 588 on bla_{CTX-M} gene expression of ESBL-producing *E.coli* Tohoku 9089. □: control (culture supernatant 0%); ▭: 59% of *C. butyricum* culture supernatant; ■: 100% of *C. butyricum* culture supernatant. Data were shown as mean ± SD. *: $p < 0.05$ vs Control.

3.4. Effects of gut microbiota and probiotic strain on the transmissibility of ES β-lactamase gene

Effects of gut microbiota and probiotic *C. butyricum* MIYAIRI 588 on the transmissibility of β-lactamase gene from ESBL-producing *E. coli* donor strain (Tohoku 9089) to recipient strain was shown in Table 2. The transmissibility of antibiotic resistant gene from ESBL-producing *E. coli* to *E. coli* TUM 5480 as a recipient strain was inhibited by the *C. butyricum*, *C. difficile*, *C. perfringens* and *E. faecium* supernatant but not by the *B. fragilis*, *B. longum* and *L. plantrum* supernatant ($p < 0.05$). *C. butyricum* MIYAIRI 588 also showed the inhibitory effect on the transmissibility of antibiotic resistant gene from carbapenem-resistant *K. pneumoniae* strain no.6 (Table 3).

4. Discussion

Emergence of antibiotic resistance began even before the first antibiotic β-lactam and penicillin were developed [23]. To date, ESBL-producing organisms with strong resistance against third generation cephalosporins are one of the biggest problems in the field of infectious diseases [2] and were already spread widely [24].

Luvsansharav et al. [25] reported that the patients in the nursing homes might serve as a reservoir of ESBL-producing organisms where the horizontal transfer of the ESBL producing organisms occur as evidenced by the fact that CTX-M type ESBL-producing organisms were isolated from fecal specimens.

However, the relationship between endogenous microbiota and ESBL-producing organisms has not been explored extensively yet. In this study, we first examined inhibitory and/or promotive activities of several gut microbiota on the growth of ESBL-producing *E. coli* strain and the results showed that *C. butyricum*, *C. difficile*, *C. perfringens*, *E. faecium* and *L. plantarum* suppressed the growth of *E. coli*.

The bacterial antagonistic action may be mediated by the production of substances by each strain such as lactic, acetic, propionic and butyric acids, hydrogen peroxide, bacteriocins, bacteriocin-like substances and possibly biosurfactants, which are known to act against the growth of certain pathogens [26]. Especially, as Lawley and Walker [27] described in their review, short chain fatty acid produced by gut microbiota may attribute to intestinal colonization resistance of the pathogenic bacteria. Another study also demonstrated that short chain fatty acid such as butyric, acetic and propionic acids inhibited the growth of enterohaemorrhagic *E. coli* O157:H7 at low acidic pH ($pH < 6$) as similar in the normal colon where the pH level was sustained at neutral pH by homeostasis [28].

The present study demonstrated that *C. butyricum* strongly inhibited the growth of ESBL-producing *E. coli* by both supernatant ($p < 0.05$) as shown in Fig. 1 and co-culture ($p < 0.05$) assays as shown in Fig. 2 and Table 1. Therefore, it is plausible that the inhibitory effect of *C. butyricum* MIYAIRI 588 on the growth of ESBL-producing *E. coli* might be derived from the production of an antagonistic substance(s) such as butyric acid, as *C. butyricum* MIYAIRI 588 has been known to secrete. However, these kinds of inhibitory activity might not specifically demonstrate to the ESBL-producing *E. coli* since *C. butyricum* MIYAIRI 588 has reported similar effect to the other antibiotic sensitive enterohaemorrhagic *E. coli* [11], and for the other microbiota strains, we might need further experiences. In any case, it is possible that the presence of certain gut microbiota may reduce the number of ESBL-producing *E. coli* in the intestinal tract of reservoir as well as the risk on horizontal transfer.

Probiotics have been suggested as an alternative therapy for the treatment of infectious gastroenteritis or for the prevention of antibiotic-associated diarrhea. These non-pathogenic microbiota are known to have beneficial effects on the digestive ecosystem and to confer resistance to infections as demonstrated by studies in animal models as well as by clinical trials for the use of biotherapeutic agents [7]. However, it has not been reported till now regarding the synergistic treatment effect of combined use of probiotics and antibiotics for the infectious diseases including ESBL-producing *E. coli* infection.

It is well known that compounds such as clavulanic acid can inactivate β-lactamase activities [29] and are commonly used for the treatment of β-lactamase positive antibiotic resistant bacterial

Table 2
Effects of culture supernatant of intestinal microorganisms on conjugative transfer frequency of *bla* genes of ESBL-producing *E. coli* Tohoku 9089.

Bacterial strain	Number of bacteria (log cfu/ml)			Transfer frequency (log)	
	Donor	Recipient	Trans-conjugant	per Donor	per Recipient
Control	9.13 ± 0.22	8.20 ± 0.38	3.70 ± 0.44	−5.28 ± 0.22	−4.35 ± 0.38
B. fragilis ATCC25285	9.49 ± 0.25*	8.19 ± 0.69	3.90 ± 0.22	−5.55 ± 0.25*	−4.25 ± 0.69
B. longum JCM1217	8.93 ± 0.18	8.36 ± 0.11	3.48 ± 0.35	−5.34 ± 0.18	−4.77 ± 0.10*
C. butyricum ATCC 19398	8.81 ± 0.30*	8.37 ± 0.10	3.01 ± 0.47*	−5.59 ± 0.30*	−5.15 ± 0.10*
C. butyricum MIYAIRI 588	8.57 ± 0.08*	8.23 ± 0.02	2.87 ± 0.18*	−5.68 ± 0.08*	−5.34 ± 0.02*
C. difficile ATCC9689	8.92 ± 0.17	8.33 ± 0.10	2.93 ± 0.20*	−5.94 ± 0.17*	−5.36 ± 0.10*
E. faecium JCM5804	8.92 ± 0.22	8.44 ± 0.06	3.16 ± 0.30*	−5.67 ± 0.22*	−5.18 ± 0.06*
L. plantarum JCM1149	8.71 ± 0.10*	8.05 ± 0.17	3.55 ± 0.43	−5.01 ± 0.10*	−4.35 ± 0.17

Donor: *E. coli* 9089 strain, Recipient: *E. coli* TUM 5480 strain, Medium: BHI, Culture supernatant: 100%.
Data are shown as mean ± SD.
*: $p < 0.05$ vs Control.

Table 3
Effects of culture supernatant of *C. butyricum* MIYAIRI 588 on conjugative transfer frequency of *bla* genes of ESBL-producing *E. coli* and CRE.

Donor	Conc. of sup.	Number of bacteria (log cfu/ml)			Transfer frequency (log)	
		Donor	Recipient	Trans-conjugant	per Donor	per Recipient
E. coli 9089	0% (Control)	8.91 ± 0.02	8.30 ± 0.02	3.89 ± 0.15	−5.00 ± 0.02	−4.39 ± 0.02
	50%	8.72 ± 0.08*	8.11 ± 0.12	3.69 ± 0.36	−4.94 ± 0.08	−4.33 ± 0.12
	100%	8.57 ± 0.08*	8.23 ± 0.02*	2.87 ± 0.18*	−5.68 ± 0.08*	−5.34 ± 0.02*
K. pneumoniae strain no.5	0% (Control)	8.85 ± 0.06	8.80 ± 0.05	5.07 ± 0.04	−3.78 ± 0.06	−3.72 ± 0.05
	50%	8.78 ± 0.11	8.83 ± 0.06	4.99 ± 0.04	−3.79 ± 0.11	−3.84 ± 0.06
	100%	8.71 ± 0.05*	8.94 ± 0.09	5.01 ± 0.02	−3.71 ± 0.05	−3.93 ± 0.09*

Recipient: *E. coli* TUM 5480 strain, Medium: BHI, Culture supernatant: *C. butyricum* MIYAIRI588.
Data are shown as mean ± SD.
*: $p < 0.05$ vs Control.

infection. However, such β-lactamase inhibitor is not often used as a primary treatment with infectious diseases and consequently there is a risk in distress of infectious disease treatment by ESBL producing bacteria.

In our present study, we demonstrated that the β-lactamase gene expression of ESBL-producing *E. coli* was significantly reduced by *C. butyricum* MIYAIRI 588 culture supernatant at 24 h compared to the negative control ($P < 0.05$) (Fig. 3). Furthermore, when the ESBL producing *E.coli* was inoculated to culture supernatant of *C. butyricum* MIYAIRI 588 strain, not only the activity of β-lactamase per 1 ml of medium but also the activity of β-lactamase per the protein content were lower than those of negative control ($p < 0.05$) (Figs. 1 and 2).

These results suggest that the β-lactamase activity was suppressed by substances produced and secreted by *C. butyricum* MIYAIRI 588 strain. Furthermore, the β-lactamase gene expression by qRT-PCR assay as shown in Fig. 3 demonstrated that the number of β-lactamase gene transcripts were decreased remarkably 3 h after inoculation of ESBL producing *E. coli* in supernatant of *C. butyricum* MIYAIRI 588 strain in a dose dependent manner ($p < 0.05$). Then, during the proliferation phase of *E. coli*, the β-lactamase gene expression was increased as compared with that of 3-h after inoculation; However, the β-lactamase gene expression was still relatively lower when incubated in the supernatant of *C. butyricum* MIYAIRI 588 strain as compared with that of the control group.

The previous research showed that the precursor of β-lactamase is first synthesized to secret and needs to be activated to gain its enzymatic activity [30]. It is possible that the suppression of β-lactamase activity we observed in this study may be derived from two modes of actions: the inhibitory action on the conversion from precursor β-lactamase to active β-lactamase and/or the inhibitory action on the protein synthesis of precursor β-lactamase. Thus, we conducted protein analysis for the presence/absence of precursor and mature β-lactamase based on molecular sizes in supernatant of *C. butyricum* MIYAIRI 588 strain. The result showed no apparent reduction of mature β-lactamase molecule whether or not cultured in the supernatant of *C. butyricum* MIYAIRI 588 (data not shown). Therefore, it is more likely that suppression of β-lactamase activity by supernatant of *C. butyricum* MIYAIRI 588 strain might be mainly due to the suppressive activity of gene transcription as shown in Fig. 3.

One crucial problem of ESBL-producing organisms is that they can easily transmit the β-lactamase gene on plasmid DNA [31]. Chong et al. [24] reported that the emergences ESBL-producing organisms have increased in hospitalized patients by horizontal gene transfer as well as the unexpected persistence of ESBL-producing enterobacteriaceae in the fecal microbiota. However, the role of probiotics and/or gut microbiota for the inhibitory effect on the transmission of antibiotic resistance genes has not been reported yet. In our present studies, we demonstrated that some gut microbiota including *C. butyricum* MIYAIRI 588 could suppress the transmissibility of antibiotic resistance genes between ESBL-producing *E. coli* to recipient *E. coli*, indicating that certain gut microbiota may play an important role in inhibiting the spread of antibiotic resistance genes. Further research is needed to elucidate the mechanism in which the gene transfer is controlled between donor and recipient bacteria cells. Understanding such as quorum sensing signaling among bacteria may translate into clinical significance.

These findings are indicating that some of gut microbiota affect to the growth of antibiotic resistant *E. coli* and its antibiotic resistant activities. However, these results were carried under limited condition and we are planning to investigate further detailed experiments.

In addition, we reported that the *C. butyricum* probiotic strain suppresses the growth of ESBL-producing *E. coli,* reduces β-lactamase enzymatic activity as well as its gene expression, and has an inhibitory effect on transmissibility of antibiotic resistance gene *in vitro*. These findings substantiate the use of probiotics at the time of antibiotic treatment of clinical infections. We are currently conducting further studies on the effects of probiotics on ESBL-producing bacteria.

Conflicts of interest

Authors K. Wada, K. Oka, M. Takahashi are employees of Miyarisan Pharmaceutical Co., Ltd. and may have conflict of interest. The other authors have no conflict of interest to declare.

Author's contribution

Author H. Kunishima and N. Ishibashi has equally contribution to this work. Authors H. Kunishima, N. Ishibashi, K. Wada, K. Oka, M. Takahashi, Y. Yamasaki, T. Aoyagi and H. Takemura performed the experiments. Authors H. Kunishima, M. Takahashi, M. Kitagawa and M. Kaku conceived and designed the experiments and wrote the paper.

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