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Research paper
Anaerobes in the microbiome

The impact of *Clostridium butyricum* MIYAIRI 588 on the murine gut microbiome and colonic tissue

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ABSTRACT

Background: *Clostridium butyricum* MIYAIRI 588 (CBM 588) is a probiotic bacterium that is used as an anti-diarrheal medicine in Japan. However, the impact of this probiotic on the gut microbiome has not been fully elucidated, especially, when used with antimicrobials.
Material and methods: In an *in vivo* study, CBM 588 monotherapy, clindamycin monotherapy, CBM 588 and clindamycin (combination therapy), or normal saline (control) was orally administered to mice for 4 days, and fecal samples were collected for 18 days to enumerate *C. butyricum*. We also extracted DNA from these fecal samples for metagenomics analysis by amplification of the V3-V4 region of the bacterial 16S rRNA gene and MiSeq Illumina sequencing. In addition, the concentrations of some short chain fatty acids were assessed in the fecal samples. A histological analysis was also conducted.
Results: On day 4 (the last treatment day), there was no difference in the total counts of *C. butyricum* between the CBM 588 monotherapy and combination therapy groups (5.21 ± 0.78 vs. 5.13 ± 0.45 $\log_{10}$ cfu/g, $p = 0.86$). Clindamycin treatment resulted in dramatic increases in the phylum *Firmicutes*, especially *Enterobacteriaceae*, *Clostridiaceae*, *Lactobacillus*, and *Enterococcus*, compared with the other groups during the treatment period. CBM 588 treatment modified the bacterial community composition at lower phylogenetic levels. Some bacterial taxa, such as *Bifidobacterium*, *Coprococcus*, and *Bacteroides*, were significantly increased in the combination therapy group when compared with the other groups. In the metabolic analysis, CBM 588 enhanced lactic acid production. It also enhanced the efficiency of lactic acid use for the production of butyric acid. Only the clindamycin monotherapy group showed abnormal colon tissue, with superficial epithelial necrosis and the presence of inflammatory cells.
Conclusion: CBM 588 treatment modulated the gut microbiota composition under dysbiosis due to the use of an antimicrobial with strong activity against anaerobes and significantly reduced epithelial damage.

1. Introduction

Diarrhea is a frequent adverse effect of antimicrobial use. The incidence of antimicrobial-associated diarrhea (AAD) varies between 5% and 25% among antimicrobials [1]. The cause of AAD is disturbance of the composition of the normal intestinal flora, leading to the overgrowth of pathogenic microorganisms, and the allergic and toxic effects of the antimicrobial on the intestinal mucosa or pharmacologic effects on intestinal motility. Thus, probiotics have been used for the treatment and prophylaxis of AAD [2,3].

Probiotics affect the interaction between resident microorganisms and the physiological functions of the host. *Clostridium*

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butyricum MIYAIRI 588 (CBM 588) is probiotic bacterium that is used as an anti-diarrheal medicine in Japan. CBM 588 has been reported to both prevent and cure diarrhea [4–6]. *C. butyricum* is a strictly anaerobic Gram-positive bacillus and a common human and animal gut commensal bacterium that is also frequently found in the environment. *C. butyricum* can produce endospores, which is a key characteristic related to its high tolerance to antimicrobials and gastric acid in the intestinal tract. Thus, CBM 588 may be not affected by antimicrobials, which may provide an advantage over other probiotics. However, few studies have evaluated the effects of CBM 588 on the human microbiome, especially with concomitant administration of an antimicrobial that has activity against anaerobes.

The intestinal tract harbors a complex microbial community that plays a key role in nutrition and health. The intestinal microbiome has a symbiotic relationship with the host. In certain age categories, the microbial diversity in the intestine remains stable in healthy individuals [7]. However, a shift in the microbial composition, referred to as dysbiosis, has been described in several pathologies, and alternations of the gut microbiome are believed to underlie the development of AAD. Such changes in the gut microbiome allow the outgrowth of certain pathogens, such as *C. difficile* and *C. perfringens* [8,9].

The gut microbiome also aids in digestion. Alteration of the gut microbiome can lead to significant changes in the colonic environment caused by variation in the concentration and distribution of organic compounds, such as carbohydrates, short chain fatty acids (SCFAs), and bile acids [3]. In a previous *in vitro* study simulating the luminal contents of the proximal colon, clindamycin reduced the number of anaerobes (*Clostridium* and *Bacteroides* species). This led to a reduction in the concentration of SCFAs [10]. Various metabolites produced by the resident microbiome play a significant role in host physiology. Hence, decreased bacterial carbohydrate metabolism in the colonic mucosa may result in a functional disturbance.

Despite the importance of the gut microbiome, there are few reports on the modulating effects of CBM 588 on the gut microbiome and metabolism. In this study, we examined the effects of CBM 588 on the murine intestinal microbiome through multi-omics approaches (genomics and metabolomics), and evaluated the histopathological changes to explain the mechanisms underlying the effects of CBM 588.

2. Material and methods

Material. CBM 588 bacterial powder was used for the *in vivo* studies. It is composed of 2.2×10^{10} cfu/g (Lot 61GT; MIYARISAN Pharmaceutical Co., Ltd.). Clindamycin for injection was purchased from Pfizer Japan, Inc.. Immediately before each *in vivo* experiment, the CBM 588 powder was weighed and reconstituted with sterile water. Clindamycin was diluted with appropriate diluents to achieve the desired concentration. Clindamycin solution was stored under refrigeration and discarded 12 h after reconstitution.

Animals and housing. Specific-pathogen-free, female ICR mice (9–10 weeks old) weighing approximately 30 g were obtained from Charles River Laboratories Japan, Inc. The mice were maintained and utilized according to National Research Council recommendations and were provided food and water *ad libitum*. The study was reviewed and approved by the ethics committee of Aichi Medical University.

Experimental set up. Twenty female ICR mice were divided into four treatment groups as follows (n = 5): (i) control group, (ii) clindamycin monotherapy group, (iii) CBM 588 monotherapy group, and (iv) combination therapy group (CBM 588 + clindamycin). CBM 588 was administered by oral gavage at 500 mg/kg per day (3.4×10^8 cfu/kg per day). Clindamycin was also administered by oral gavage at 40 mg/kg per day. CBM 588 powder was dissolved in sterilized water to prepare a suspension. After mixing the suspension sufficiently, the mice were forced to drink it by using sonde. For the combination group, CBM 588 and clindamycin were dissolved in sterilized water separately. A suspension containing half the daily dose was administered twice a day, at 10 a.m. and 4 p.m., for 4 days.

Assessment of physiological condition. Weight loss and stool consistency were assessed daily to determine any physical changes. Body weights were recorded every other day. These data are reported as the percentage of weight lost compared to the initial body weight.

Enumerating *C. butyricum* in feces. *C. butyricum* was enumerated in samples obtained before treatment (day 0), on the second (day 2) and fourth (day 4) days of treatment, and on the second (day 6), fourth (day 8), sixth (day 10), eighth (day 12), 10th (day 14), 12th (day 16), and 14th (day 18) days after treatment termination. On each sampling day, at least 0.3 g of feces were collected and placed into 0.45 ml of transport medium.

To determine the concentration of fecal *C. butyricum*, *C. butyricum* selective medium was used [11]. The fecal specimens were serially diluted (10 fold) to 10^5 with culture medium, spread on the selective agar plates, and incubated for 24 h. Then, the *C. butyricum* colonies were counted. The viable count, that is the number of colonies growing on the plates, was converted to the number of bacteria per gram of feces. Additionally, we exposed undiluted fecal specimens (0.05 ml) to ethanol. Then, the same process was used to determine the numbers of *C. butyricum* spores in the fecal samples. The detection limit was 2.0 ($\log_{10}$ cfu) per gram of feces.

DNA extraction. To characterize the microbiome composition in the colon, fecal samples from each mouse were analyzed by sequencing the V3–V4 regions of the 16S rRNA gene. DNA extraction and 16S rRNA sequencing were conducted as previously described [12]. The fecal contents from the mice were immediately suspended in buffer containing 4 M guanidium thiocyanate, 100 mM Tris-HCl (pH 9.0), and 40 mM EDTA (pH 8.0) and stored at 4 °C until use. For use, the fecal pellets were suspended in 10 mM Tris-HCl and 10 mM EDTA buffer (pH 8.0). Next, lysozyme (final concentration, 15 mg/mL; Sigma) was added, and the samples were incubated at 37 °C for 1 h. Then, purified achromopeptidase (Wako) was added to a final concentration of 2000 U/mL and incubated at 37 °C for 30 min. Next, SDS (final concentration: 1%) was added to the cell suspension and mixed well. Then, proteinase K (Merck) was added (final concentration: 1 mg/mL) to the suspension, and the mixture was incubated at 55 °C for 1 h. High-molecular mass DNA was isolated and purified by phenol/chloroform extraction, ethanol precipitation, and finally polyethylene glycol precipitation.

Gut microbiome analysis. Meta 16S rRNA Gene Sequencing PCR was performed by using Ex Taq Hot Start (TAKARA) and the Illumina forward primer 50-AATGATACGGCGACCACCGAGATCTACAC (adaptor sequence) + barcode (eight bases) + ACACTCTTTCCC TACACGACGCTCTTCCGATCT (sequence primer) + CCTACGGGNG GCWGCAG-30 (341F) and the Illumina reverse primer 50-CAAG-CAGAAGACGGCATACGAGAT (adaptor sequence) + barcode (eight bases) + GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT (sequence primer) + GACTACHVGGGTATCTAATCC-30 (805R) to amplify the hypervariable V3–V4 region of the 16S rRNA gene. Amplicons generated from each sample were subsequently purified using SPRI select (Beckman Coulter). The amount of DNA was quantified using the QuantiFluor dsDNA System and a Quantus Fluorometer (Promega). Mixed samples were prepared by pooling approximately equal amounts of each amplified DNA and sequenced using the MiSeq Reagent Kit V3 (600 cycle) and a MiSeq sequencer