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Microbial/host interactions

Safety evaluation of probiotic bifidobacteria by analysis of mucin degradation activity and translocation ability

Fumiaki Abe [a,*], Masamichi Muto [a], Tomoko Yaeshima [a], Keiji Iwatsuki [a], Hiroaki Aihara [b], Yuji Ohashi [b], Tomohiko Fujisawa [b]

[a] Food Science & Technology Institute, Morinaga Milk Industry Co., Ltd., 1-83, 5-Chome Higashihara, Zama-City, Kanagawa Prefecture 228-8583, Japan
[b] Laboratory of Food Hygiene, Nippon Veterinary and Life Science University, Tokyo 180-8602, Japan

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ABSTRACT

Although probiotic-containing nutrient formulas for infants and toddlers have become very popular, some adverse effects related to translocation of probiotic strains have been reported. We assessed the safety of probiotic bifidobacteria that have been used in clinical investigations and proven to have beneficial effects, by analyzing mucin degradation activity and translocation ability. Mucin degradation activities of three probiotic bifidobacteria strains; *Bifidobacterium longum* BB536, *Bifidobacterium breve* M-16V and *Bifidobacterium infantis* M-63, were evaluated by three *in vitro* tests comprising growth in liquid medium, SDS-PAGE analysis of degraded mucin residues, and degradation assay in Petri dish. All test strains and control type strains failed to grow in the liquid medium containing mucin as the only carbon source, although good growth was obtained from fecal sample. In the SDS-PAGE analyses of mucin residues and observation of mucinolytic zone in agar plate, the three test strains also showed no mucin degradation activity as the type strains, although fecal sample yielded positive results. In another study, a high dose of *B. longum* BB536 was administered orally to conventional mice to examine the translocation ability. No translocation into blood, liver, spleen, kidney and mesenteric lymph nodes was observed and no disturbance of epithelial cells and mucosal layer in the ileum, cecum and colon was detected, indicating that the test strain had no translocation ability and induced no damage to intestinal surface. These results resolve the concern about bacterial translocation when using bifidobacteria strains as probiotics, which have been tested in various clinical trials, supporting the continuous use of these probiotic strains without anxiety.

1. Introduction

Many scientists have studied and reported the positive effects of bifidobacteria on human health [1–6]. Now bifidobacteria are known to be one of the most beneficial bacteria and are used globally as probiotics in various food products including yogurt, milk, infant formula, cheese, and dietary supplement [7–11]. Also some bifidobacterial strains including *Bifidobacterium longum* BB536 are used in Japan as a functional ingredient in products designated as Food for Specified Health Uses (FOSHU) [12].

On the other hand, safety issues of probiotics have been discussed [13–15]. Snydman [15] listed three theoretical concerns regarding safety of probiotics: the occurrence of disease, toxic or metabolic effect on the gastrointestinal tract, and the transfer of antibiotic resistance in the gastrointestinal flora. The above reports raised the necessity of investigating not only the beneficial effects but also safety of probiotics on human health. In particular, the potential of causing diseases such as bacteremia, endocarditis and sepsis is assumed to be a serious risk for probiotics, because some investigators reported *Lactobacillus* strain isolated from blood samples was indistinguishable from the probiotic strain ingested by the patients, indicating a possibility of bacterial translocation of the probiotic strain [16–18]. The first step of bacterial translocation is the invasion of bacteria through the intestinal wall. Gork et al. [19] have reported that mucin on the surface of the intestinal wall is very important to prevent bacterial translocation. Also, mucin degradation activity has been used as an index of safety of probiotic strains in previous reports [20,21].

Many clinical studies have investigated various effects of *B. longum* BB536 and *B. breve* M-16V strains on human health, and harmful incidents have never been reported. Puccio et al. [22] and Chouraqui et al. [23] administered infant formula supplemented

* Corresponding author. Tel.: +81 46 252 3047; fax: +81 46 252 3055.
E-mail address: f_abe@morinagamilk.co.jp (F. Abe).

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with *B. longum* BB536 to infants and evaluated the safety and tolerance; both studies confirmed that the formulas showed no harmful effects. Li et al. [24] and Fujii et al. [5] administered *B. breve* M-16V to low-birth-weight infants. They confirmed the effectiveness of this strain on the formation of healthy intestinal microflora and observed no adverse event. Furthermore, *B. longum* BB536 has been used as a probiotic ingredient for probiotic milk or yogurt since 1977 in Japan, without any report of adverse effect during more than 30 years of use as a commercial product. From the above information, it has been assumed that both *B. longum* BB536 and *B. breve* M-16V are safe not only for adults but also for infants. However, despite the many clinical studies and long history of use, basic research for safety evaluation is important to ensure that probiotic strains have no potential risk.

The primary purpose of the present study was to evaluate the safety of *B. longum* BB536 and *B. breve* M-16V, which have been studied for their clinical effects, by conducting in vitro mucin degradation tests and in vivo translocation test using conventional animals. Also, considering the safety of the genus *Bifidobacterium*, *B. infantis* M-63 was used as an additional test strain in the safety tests.

2. Materials and methods

2.1. Bifidobacteria strains

Three bifidobacterial strains comprising *B. longum* subsp. *longum* BB536 (*B. longum* BB536), *Bifidobacterium breve* M-16V and *B. longum* subsp. *infantis* M-63 (*B. infantis* M-63) were used. All strains were isolated from infants and deposited into culture collections; *B. longum* BB536 as American Type Culture Collection strain BAA-999, *B. breve* M-16V as Belgian Co-Ordinated Collections of Micro-organisms (BCCM) strain LMG23729, and *B. infantis* M-63 as BCCM strain LMG23728. Also, the corresponding type strains; *B. longum* ATCC 15707, *B. breve* ATCC 15700 and *B. infantis* ATCC 15696, were purchased from American Type Culture Collections and used as control strains. In mucin degradation tests, each strain cultured in MRS medium (OXOID, Cambridge, UK) at 37 °C for 24 h under anaerobic condition was used. In the present study, anaerobic condition was achieved using Anaero Pack (Mitsubishi Gas Chemical Co., Tokyo, Japan). In bacterial translocation test, lyophilized *B. longum* BB536 manufactured at Morinaga Milk Industry Co., Ltd. (Tokyo, Japan) was used. The bifidobacterial cell counts were analyzed by previous report [25].

2.2. Mucin degradation tests

Mucinolytic activity was examined using three tests: growth in liquid medium, SDS-PAGE analysis of degraded mucin residues, and degradation assay in Petri dish, according to previous reports with slight modifications [20,21]. In all mucin degradation tests, partial purified hog gastric mucin (HGM) from a commercial source (type III, Sigma–Aldrich, Inc., MO, USA) was used after further purification. Fecal sample collected from healthy adult and the autoclaved fecal sample (121 °C, 20 min) were used as positive and negative control, respectively. SDS, amido black, acetic acid, and other chemical reagents were purchased from (Wako Pure Chemical Industry Ltd, Osaka, Japan).

Briefly, 100 μl of MRS culture was inoculated into 10 ml of basal medium containing 0.3% HGM with or without 1% glucose, and cultured at 37 °C for 48 h under anaerobic condition (Anaero Pack). Composition of the basal medium was 1 g of Bacto™ Peptone (Becton Dickinson, NJ, USA), 1 g of Trypticase™ Peptone (Becton Dickinson, NJ, USA), 2 g of yeast extract (Becton Dickinson, NJ, USA), 0.1 g of L-cysteine-HCl, 4 ml of mineral solution-1 (0.78% K_2HPO_4 solution), 4 ml of mineral solution-2 (0.47% KH_2PO_4, 1.18% NaCl, 1.2% $(NH_4)_2CO_4$, 0.12% $CaCl_2$, 0.25% $MgSO_4 \cdot H_2O$), 3 ml of Fildes solution (digestive blood solution), and 189 ml of distilled water. The Fildes solution is often used as a supplement for the culture of anaerobic bacteria related to intestinal microflora [26]. After incubation, bacterial growth was assessed by measuring absorbance at 600 nm (Hitachi Spectrophotometer; Hitachi High-Technologies Co., Tokyo, Japan) and pH of the culture. Each test was performed in triplicate and the results were presented in mean ± standard deviation (SD).

The basal medium culture of each strain was used in SDS-PAGE. At the end of incubation, 10 ml of culture was centrifuged (10,000 × g, 4 °C, 30 min) to obtain a cell-free supernatant. The supernatant was mixed with 15 ml of 99% chilled ethanol and re-centrifuged (10,000 × g, 4 °C, 30 min). The pellet was collected and suspended in 6 ml of 0.1 M NaCl. Ethanol precipitation and re-centrifugation were repeated two times to purify the mucin. Finally the pellet was resuspended with 0.5 ml of 10 mM Tris–HCl buffer and used as the SDS-PAGE sample. To demonstrate any change in the composition of mucin after incubation in liquid medium, the electrophoretic patterns of ethanol-precipitated mucin samples were analyzed by SDS-PAGE using 12.5% polyacrylamide as the separating gel. Gels were stained with Coomassie blue (Bio-Safe™ Coomassie, Bio-Rad Laboratories, Inc., USA), for protein pattern, and with both Coomassie blue and PAS stain (GelCode® Glycoprotein Staining Kit, Thermo Scientific, IL, USA) for glycoprotein pattern. Any de novo band with a smaller molecular weight compared with the negative control (autoclaved feces) was defined as positive mucin degradation.

Mucin degradation assay in a Petri dish was performed according to previous report [20]. Briefly, agar plate medium prepared from the basal medium supplemented with 1.5% agar (Becton Dickinson, NJ, USA), 0.3% HGM, with or without 1% glucose was used for the test. Ten μl of MRS culture were inoculated on the surface of the agar plate in a Petri dish. The plates were incubated at 37 °C anaerobically for 72 h and subsequently stained with 0.1% amido black in 3.5 M acetic acid for 30 min, and then washed with 1.2 M acetic acid. Mucin lysis zone (discolored halo) around the colony was observed.

2.3. Translocation test

Bacterial translocation ability of *B. longum* BB536 was determined using 4-week-old mice (BALB/cAnNCrlCrlj, SPF) purchased from Charles River Japan Inc. (Tokyo, Japan). Five male and five female mice each were allocated to a test (*B. longum* BB536) group and a control group. Each group of five animals was housed in a stainless steel cage in a controlled environment (temperature 20–24 °C, humidity 45–75%) with 12-h light/dark cycle, and allowed free access to a sterilized pellet diet (Funabashi Farm Co., Ltd., Chiba, Japan) and water during the experimental period.

After 7 days of acclimation, bifidobacteria powder suspended in saline was administered to mice. Test animals were administered 9.3×10^{11} CFU/kg/day of *B. longum* BB536 orally using a stomach tube once a day for seven days. Mice in control group were administered potato starch solution instead of bifidobacteria powder. Observation of general signs and body weight measurement was conducted during the test period. On the eighth day which was one day after the final administration, all mice were sacrificed after blood was collected from the heart under anesthesia. After gross observation for abnormalities in all organs and tissues, the liver, spleen, kidney, ileum, cecum, colon and mesenteric lymph node were collected under biological clean condition for bacterial translocation analysis and histopathological examination.

For bacterial translocation analysis, each organ was minced with sterilized scalpel and the minced tissues were spread on BL agar plates (Nissui Co., Ltd, Tokyo, Japan) used for intestinal microbiota analysis [27]. In case of blood, 0.2 ml blood was spread on the