Bioscience of Microbiota, Food and Health Vol. 35 (3), 137–140, 2016

Protective effects of heat-killed *Lactococcus lactis* subsp. *lactis* BF3, isolated from the intestine of chum salmon, in a murine model of DSS-induced inflammatory bowel disease

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Received March 9, 2016; Accepted April 6, 2016; Published online in J-STAGE April 19, 2016

Oxidative stress is considered an etiological factor responsible for several symptoms of inflammatory bowel disease (IBD). *In vitro* anti-inflammatory activities of heat-killed *Lactococcus lactis* subsp. *lactis* BF3 have been reported. In this study, the anti-inflammatory effect of these cells was examined using a dextran sodium sulphate (DSS)-induced murine IBD model. Administration of heat-killed *L. lactis* BF3 via drinking water suppressed the IBD symptoms, such as shortening of colon length, damage to the colon mucosa as observed under the microscope, and spleen enlargement. This result suggests that heat-killed *L. lactis* BF3 has the potential to treat IBD.

Key words: *Lactococcus lactis*, inflammatory bowel disease, mice

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD). Although IBD is thought to be primarily a condition of young individuals, a significant proportion of new cases of IBD are diagnosed in elderly persons [1]. Recently, it was estimated that over 1 million residents in the USA and 2.5 million residents in Europe have IBD [2]. Moreover, IBD has increased in Asia, South America, and the Middle East including newly industrialized countries [2, 3].

The pathogenesis of IBD is not completely understood, but it is considered that oxidative stress is an etiological factor involved in several signs and symptoms of IBD [4]. Therefore, many studies have focused on describing food materials and extracted compounds with antioxidant activities to be used for IBD treatment. Recently, the amelioration of IBD by some lactic acid bacteria (LAB), owing to their antioxidant and anti-inflammatory effects has also been studied [5, 6]. Previously, we isolated LABs from the intestine of chum salmon (*Oncorhynchus keta*) that were caught in the sea near Rausu, in Japan. Among the LAB isolates, *Lactococcus lactis* subsp. *lactis* BF3 showed higher acid, bile, and salt resistances compared with those of the type strain [7]. Furthermore, heat-killed cells of *L. lactis* BF3 showed a protective effect in eukaryotic cells, protecting them from hydrogen peroxide toxicity and the inhibitory effect of *E. coli* lipopolysaccharide (LPS)-induced nitric oxide (NO) secretion in murine macrophage RAW264.7 cells. These results suggest that not only live cells but also heat-killed cells of *L. lactis* BF3 have potential to be used for IBD treatment through stress resistance and antioxidant and anti-inflammatory activities. However, these effects of *L. lactis* BF3 have not been confirmed *in vivo*. In this study, to verify the effectiveness of heat-killed *L. lactis* BF3 against IBD, the anti-inflammatory effect of these cells in a dextran sodium sulphate (DSS)-induced murine model of IBD was examined.

L. lactis BF3 (accession No. AB973593) isolated from *Oncorhynchus keta* was stored in a Microbank (Iwaki Co., Tokyo, Japan) at −80°C [7]. Before examination, the frozen strains were thawed and pre-cultured in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) at 37°C for 24 hr. The bacterial cells were washed with PBS three times, suspended in distilled water,

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Table 1. Body and organ weights of the experimental mice

	Control	DSS control	BF3
Body weight (g)			
Initial	29.7 ± 0.6	30.1 ± 0.5	30.3 ± 0.5
3 days after BF3 administration	31.2 ± 0.4	31.8 ± 0.7	31.6 ± 0.6
7 days after DSS administration	33.7 ± 0.5	30.8 ± 1.2	31.7 ± 1.0
Organ weight (g)			
Liver	1.916 ± 0.041[a]	1.414 ± 0.082[b]	1.580 ± 0.082[b]
Kidneys	0.515 ± 0.016	0.510 ± 0.018	0.513 ± 0.026
Spleen	0.117 ± 0.098[b]	0.244 ± 0.046[a]	0.146 ± 0.015[ab]

Values are shown as the mean and SEM (n=6).
Means within each SEM having different letters are significantly different ($p<0.05$).

adjusted to an OD_{660} of 10 (about 10^9 colony forming units (CFU)/ml), and subjected to heat treatment in boiling water for 20 min. The prepared cell suspension was stored at 4°C and used within 3 days.

This animal experiment was performed in compliance with the fundamental guidelines for proper conduct of animal experiments and related activities in academic research institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan. It was approved by the animal experiment committee of the Tokyo University of Marine Science and Technology (Approval No. H27-4).

Eighteen 5-week-old male ddY mice were obtained from Tokyo Laboratory Animals Science (Tokyo, Japan). The mice were acclimatized in a negative pressure rack maintained at 20–24°C, with a relative humidity of 50–60%, and they were fed a CE2 diet (CLEA Japan, Tokyo, Japan) and distilled water. After 7 days, the mice were divided into 3 groups (n=6). Among them, the untreated control (control) and DSS control groups were fed the same diet and distilled water. The BF3 treated group was fed the same diet but was fed the prepared *L. lactis* BF3 cell suspension instead of drinking water. After 3 days, 5% (w/v) DSS (MW = 5000; Wako Pure Chemical Industries, Osaka, Japan) was added to the drinking water of the DSS control and BF3-treated groups. The mice received diet and water *ad libitum*.

After 7 days of DSS administration, the mice were anesthetized with diethyl ether and exsanguinated from the abdominal aorta. The large intestine (colon) was excised and washed with PBS, and then its length was measured. The severity of IBD was also evaluated on the basis of histological observations of hematoxylin and eosin (HE)-stained tissues of the colon [6]. Approximately 5-mm-long sections of the middle section of the colon were soaked in 10% formalin to prepare samples for microscopic analysis and HE staining (MedRidge, Tokyo, Japan). The liver, spleen, and kidneys were also excised and weighed as indices of DSS toxicity [8, 9].

Data are presented as the mean ± SEM. Data from experiments were analyzed by ANOVA and Tukey's test using a statistical software (Excel Statistic Ver. 6, Esumi, Tokyo, Japan).

To determine the anti-inflammatory effect of heat-killed *L. lactis* BF3 on IBD, 5% (w/v) DSS in drinking water was administered to mice with or without treatment of *L. lactis* BF3. As shown in Table 1, after 7 days of DSS treatment, the body weights in the DSS control group tended to be lower than those in the control group. This effect was tended to be suppressed by the LAB cells. At that time, diarrhea and bloody bowel discharge were observed only in mice of the DSS control group.

DSS treatment decreased the liver weight from about 1.916 g to 1.414 g. This effect was also suppressed by *L. lactis* BF3. There was no significant effect on kidney weight. The weight of the spleen of mice in the DSS control group was about two times higher than that of the control group mice. The spleen enlargement also tended to be supressed by *L. lactis* BF3. Enlargement of the spleen, an organ of the immune system, caused by the administration of DSS has been previously reported [6, 9].

As shown in Fig. 1A, the colon length was shorter in mice in the DSS control group compared with that observed in the control group mice. This represents the index of inflammation caused by IBD [10]. However, treatment with *L. lactis* BF3 resulted in a recovery of colon length by approximately 50% compared with the DSS control group. This result indicates that *L. lactis* BF3 prevented IBD induced by DSS. Fig. 1B shows typical images of HE-stained colon tissue. In the control group, the sections of the crypt structure in the mucosal layer, the submucosa, and muscular layer were normal. In the DSS control group, the crypt structure and submucosa

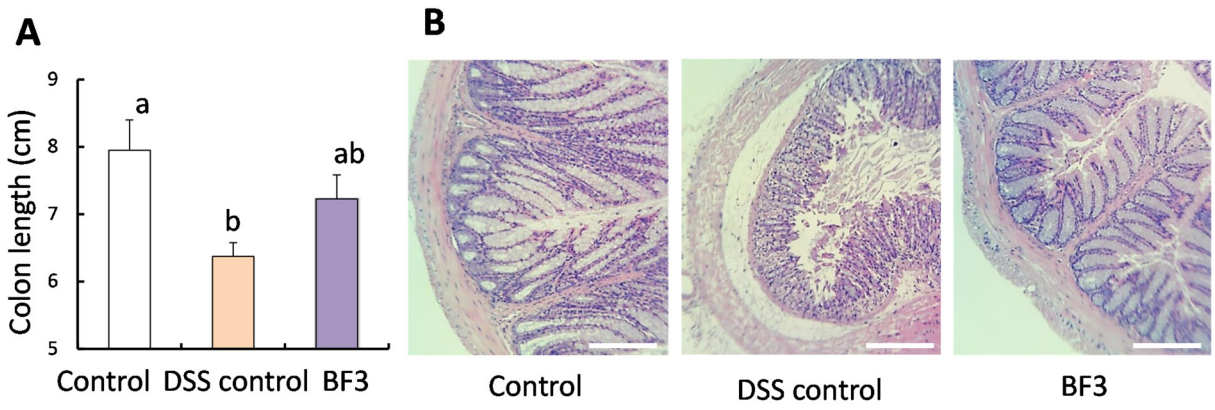

Fig. 1. Colon length (A) and images of hematoxylin and eosin (HE)-stained colons (B) of mice that drank distilled water (control), distilled water and 5% (w/v) DSS (DSS control), or DSS with distilled water containing heat-killed cells of *L. lactis* BF3 (BF3). Values in (B) are expressed as the mean ± SE (n=6). Means within each error bar having different letters are significantly different ($p<0.05$). Scale bars=0.25 mm.

were irregular. These irregularities caused by DSS were suppressed by treatment with *L. lactis* BF3.

In many studies of anti-inflammatory effects on macrophage cells and enterocytes, the heat-killed LAB cells have been used [6, 7, 11, 12]. On the other hand, the anti-inflammatory effects of live cells on a DSS-induced murine model of IBD have also been reported by many researchers [13–15]. It is thought that it may be better to use heat-killed LAB cells, as they may be more stable and safer than live cells [16]. It was previously reported that heat treatment denatured the cell membrane and outer cell compounds [17]. In some cases, the denatured cells do perform some functions as well as or better than live cells. For examples, Li et al. [16] reported that both live and heat-killed *Lactobacillus rhamnosus* GG provided suppressed LPS-induced proinflammatory mediators and increased anti-inflammatory mediators. Furthermore the *in vitro* bile acid binding capacities of cells of *Lactobacillus plantarum*, *L. lactis,* and *Leuconostoc mesenteroides* strains isolated from fish intestines were increased by heating [18]. The findings of this study suggest that the heat-killed cells of *L. lactis* BF3 also have the potential for anti-IBD therapy. Studies on functional compounds such as exopolysaccharides, and the mechanisms of the reported anti-IBD effect of live and heat-killed *L. lactis* BF3 are currently in progress.

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