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Lactococcus lactis subsp. *cremoris* FC alleviates symptoms of colitis induced by dextran sulfate sodium in mice

Yosuke Nishitani [a,*], Takeshi Tanoue [b], Katsushige Yamada [c], Tsukasa Ishida [d], Masaru Yoshida [d,e], Takeshi Azuma [d], Masashi Mizuno [b]

[a] Organization of Advanced Science and Technology, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan
[b] Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan
[c] Development Center, Fujicco Co., Ltd., 6-13-4 Minatojima-Nakamachi, Chuo-ku, Kobe 650-8558, Japan
[d] Gastroenterology Division, Department of Internal Medicine, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
[e] The Integrated Center for Mass Spectrometry, Graduate School of Medicine, Kobe University, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

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ABSTRACT

Probiotics have been used to treat human gastrointestinal inflammations including inflammatory bowel disease (IBD). However, the exact mechanisms by which probiotics act to protect against intestinal inflammation have yet to be fully elucidated. The aim of this study was to evaluate anti-inflammatory effects of *Lactococcus lactis* subsp. *cremoris* FC using *in vivo* and *in vitro* inflammation models. Colitis was induced in C57BL/6 mice by administration of 3% dextran sulfate sodium to drinking water. In the cellular level assessment, a gut inflammation model with the co-culture system consisting Caco-2 cells and RAW264.7 cells stimulated by LPS was used. Administration of *L. lactis* subsp. *cremoris* FC significantly ameliorated shortening of colon length and histological score of the colon in DSS-induce colitis mice. In addition, the treatment of *L. lactis* subsp. *cremoris* FC improved the aberrant mRNA expression in inflamed tissue near to control level through notable suppression of TNF-α ($P<0.05$), IFN-γ ($P<0.05$), IL-6, iNOS, and MIP-2 mRNA expression. In addition, in a gut inflammation model, treatment with *L. lactis* subsp. *cremoris* FC resulted in significant down-regulation of IL-8 mRNA expression in Caco-2 cells and inhibition of NF-κB nuclear translocation in RAW264.7 cells. Our findings indicate that administration of *L. lactis* subsp. *cremoris* FC improves negative effects of DSS-induced colitis in mice through the inhibition of inflammatory cell infiltration.

1. Introduction

Inflammatory bowel disease (IBD) includes Crohn's disease (CD) and ulcerative colitis (UC), both of which are characterized by chronic and relapsed inflammation of gut, but their etiology still remains unknown. It is thought that the disease might be due to the consequence of overly aggressive immune responses to enteric bacterial components in genetically predisposed individuals [1–4]. In various studies, typical features of these diseases have shown that intestinal epithelial cells and macrophages secrete large amounts of chemokines and pro-inflammatory cytokines in the inflamed intestine of IBD patients, and that modulation of the mucosal epithelial barrier played a critical role in the initiation and propagation of IBD [5,6].

Probiotic organisms have been used to treat a variety of human gastrointestinal ailments including IBD [7], irritable bowel syndrome [8], and pouchitis [9,10], as well as rotavirus and antibiotic-associated diarrhea [11–13]. Although little is known about their mechanisms of action, probiotics seem to have protective, trophic, and anti-inflammatory effects on bowel mucosa. Proposed mechanisms include the production of ammonia, hydrogen peroxide [14–16], and bacteriocins [17,18], which inhibit the growth of pathogenic bacteria, the competition for adhesion sites on intestinal epithelia [19,20], and an adjuvant-like stimulation of the immune system against pathogenic organisms [21]. However, the exact mechanisms by which probiotics act to protect against intestinal inflammation are not known.

To evaluate anti-inflammatory effects and mechanisms of the action exhibited by probiotics, *in vitro* models in a single culture consisting epithelial cells or immunocompetent cells with proper stimulants have been used [22–25]. The treatment of various probiotic strains including *Lactobacillus casei* Shirota, *Lactobacillus rhamnosus* GG, *Lactobacillus reuteri*, and VSL#3 (composed of *Streptococcus thermophilus* and several species of Lactobacilli and Bifidobacteria) resulted in inhibition of tumor necrosis factor (TNF)-α-induced interleukin (IL)-8 production from epithelial cell lines [22,25], or lipopolysaccharide (LPS)-induced pro-inflammatory cytokines production using these models [23,24]. However, epithelial cells that line the intestinal tract are considered to participate in the initiation and

* Corresponding author. Tel./fax: +81 78 803 5907.
E-mail address: nishitani@people.kobe-u.ac.jp (Y. Nishitani).

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regulation of the mucosal immune response to bacteria by interacting with immunocompetent cells such as macrophages and lymphocytes [26]. Therefore, a more precise *in vitro* assessment model of anti-inflammatory effects is required to elucidate this mechanism. As part of this requirement, we had developed a novel gut inflammatory model [27].

Probiotic effects of *Lactococcus lactis* subsp. *cremoris* FC such as improving defecation frequency and fecal microflora in healthy elderly volunteers were previously described [28]. In this study, we assessed anti-inflammatory effects of *L. lactis* subsp. *cremoris* FC on colonic mucosal damage and on the inflammatory response in a dextran sulfate sodium (DSS) colitis model. Using this experimental model, *L. lactis* subsp. *cremoris* FC showed preventive and therapeutic effects with amelioration of shortening of colon length and histological score, and an attenuation of pro-inflammatory cytokines mRNA expression in inflamed colon tissue. Moreover, in order to clarify the mechanisms of the action, we used a gut inflammation *in vitro* model co-cultured with intestinal epithelial cells and macrophages described in our previous study [27]. Treatment with *L. lactis* subsp. *cremoris* FC resulted in significant down-regulation of IL-8 mRNA expression in Caco-2 cells and inhibition of NF-κB nuclear translocation in RAW264.7 cells. These results suggest some mechanisms of action of one clinically significant probiotic organism.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM, glutamine, low glucose) and lipopolysaccharide (LPS) from *E. coli* O127 were purchased from Wako Pure Chemical Industries (Osaka, Japan). MEM (Eagle's Minimum Essential Medium) was purchased from Nissui Pharmaceutical Co. Ltd., (Tokyo, Japan). RPMI 1640 medium and MEM non-essential amino acids (NEAA) were purchased from Gibco BRL (Grand Island, NY, USA). DMEM (glutamine, high glucose) and budesonide were obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Beit, Israel). DSS (molecular weight 36,000–50,000) was purchased from MP Biomedicals (Solon, OH, USA).

2.2. Animals

Female C57BL/6 mice (6-weeks-old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were inbred under a light:dark cycle every 12 h at room temperature and fed food and water *ad libitum*. All animal experiments were approved by the Animal Experiment Ethnics Committee of Kobe University.

2.3. Bacterial strain

L. lactis subsp. *cremoris* FC was cultured in Mann Rogosa Sharp (MRS) broth and agar (Difco Laboratories, Detroit, MI, USA) and incubated overnight at 30 °C in anaerobic chamber. After incubation, cells were obtained by centrifugation (4 °C, 10,000×*g*, 5 min). Pellet was suspended in PBS and washed three times in order to remove cell culture medium. Bacterial suspensions were irradiated with an UV germicidal lamp before the experiments. After this treatment, viable counts were below 10^2 CFU/ml. This represents more than 9-log reduction of viability. Cells were stored at −80 °C until used.

2.4. Induction of acute DSS colitis

Mice were randomly divided into four groups: DSS untreated mice without administration of strain FC ($n=3$); DSS untreated mice with administration of strain FC ($n=2$); DSS treated mice without administration of strain FC ($n=6$); and DSS treated mice with administration of strain FC ($n=6$). Colitis was induced in 8 weeks old female mice by administration of 3% (w/v) DSS to drinking water. Strain FC and vehicle treatment were carried out by a daily intragastric administration of a 1×10^9 CFU dose starting 7 days prior to DSS treatment and continuing daily until sacrifice. On day 5, DSS administration was stopped, and normal drinking water was supplied for the subsequent 3 days. All mice were weighed everyday. Mice were killed on day 8. Colons were excised, and their length and thickening were documented. Histological examination was performed in a blinded manner, and the degree of inflammation and epithelial damage on microscopic hematoxylin and eosin (HE) staining sections (8 μm) of distal colon was graded according to the method of Hudert and coworkers [29]. Briefly, the inflammation score consisted of (i) severity of inflammation (0, no inflammation; 1, mild; 2, moderate; 3, severe) and (ii) thickness of inflammatory cell infiltration (0, no inflammation; 1, mucosa; 2, mucosa plus submucosa; 3, transmural) and epithelial damage score consisting of character (0, intact epithelium; 1, disruption of architectural structure; 2, erosion; 3, ulceration) and extent (0, no lesions; 1, punctuate; 2, multifocal; 3, diffuse) of lesions. The total highest score in this study was 10.

2.5. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from distal colon with Sepasol RNA I super (Nacalai Tesque, Inc., Kyoto, Japan) with High salt precipitation solution (Nippon Gene Co. Ltd., Tokyo, Japan) according to manufacturer's protocol. Reverse transcription was performed as described previously [27]. Oligonucleotide primers used for PCR amplification and the size of the PCR products obtained from target cellular RNA are shown in Table 1. After an initial incubation at 95 °C for 5 min, PCR was performed with 25 cycles for β-actin, 30 cycles for TNF-α, IL-6, and IFN-γ, 35 cycles for IL-12 p40 and iNOS, and 40 cycles for IL-4 consisting of 45 s of denaturation at 95 °C, 45 s of annealing at 58 °C, and 45 s of extension at 72 °C. The amplified products were separated by electrophoresis on a 2.3% agarose gel and visualized by ultraviolet (UV) light illumination using ethidium bromide staining. To semi-quantify the induction of TNF-α, IL-6, IL-12 p40, IFN-γ, iNOS, and IL-4

Table 1
Oligonucleotide primers and PCR product sizes used in this study.

mRNA species	Sequences (5′–3′)	Size, bp
β-actin		
Forward	5′-TGGAATCCTGTGGCATCCATGAAAC-3′	345
Reverse	5′-AACGCAGCTCAGTAACAGCTCCGCCTA-3′	
IL-12 p40		
Forward	5′-GGAGACCCTGCCCATTGAACT-3′	414
Reverse	5′-CAACGTTGCATCCTAGGATCG-3′	
TNF-α		
Forward	5′-GGCAGGTCTACTTTGGAGTCATTGC-3′	299
Reverse	5′-ACATTCGAGGCTCCAGTGAAATTCGG-3′	
IL-6		
Forward	5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′	155
Reverse	5′-TCTGACCACAGTGAGGAATGTCCAC-3′	
IFN-γ		
Forward	5′-AGCGGCTGACTGAACTCAGATTGTAG-3′	220
Reverse	5′-GTCACAGTTTTCAGCTGTATAGGG-3′	
IL-4		
Forward	5′-CGAAGAACACCACAGAGAGTGAGCT-3′	170
Reverse	5′-GACTCATTCATGGTGCAGCTTATCG-3′	
iNOS		
Forward	5′-ATGGCTTGCCCCTGGAAGTTTC-3′	308
Reverse	5′-GGACTTGCAAGTGAAATCCGATG-3′	