Effect of *Bifidobacterium bifidum* BF-1 on gastric protection and mucin production in an acute gastric injury rat model

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ABSTRACT

Homeostasis in the stomach environment is maintained by the balance of protective factors such as gastric mucus and aggressive factors such as gastric acid, stress, alcohol, and drugs. An overload of aggressive factors that upsets this balance can induce gastric injury. Fermented milk that contains *Bifidobacterium bifidum* BF-1 (BF-1), a probiotic strain, and *Streptococcus thermophilus* YIT 2021 (ST) is known to improve *Helicobacter pylori*-associated gastritis in humans. Here, we investigated the gastroprotective potential of BF-1 in a rat model of acid-ethanol-induced acute gastric injury to fully elucidate its potential compared with ST. Living BF-1, ST, or vehicle was orally administered to rats, and acid-ethanol gastric injury was induced 2 h later. The gastric injury rate was determined and shown to be significantly lower in the BF-1 group than in the vehicle group, which showed a similar level to the ST group. The production of gastric mucin and the expression of several target genes associated with protection and inflammation were examined before and after induction of gastric injury. Interestingly, mucin 5ac (*muc5ac*) gene expression in gastric corpus samples and gastric mucin production in stomach samples from the BF-1 group, but not the ST group, were significantly higher than those in the respective samples from the vehicle group. These findings indicate that BF-1 has the potential to provide gastroprotection, alleviating acute gastric injury by enhancing the production of gastric mucin in a rat model.

Key words: *Bifidobacterium bifidum* BF-1, gastroprotective effect, mucin, probiotics

INTRODUCTION

Homeostasis in the stomach is widely believed to be maintained through balancing protective factors such as gastric mucus, bicarbonates, and gastric mucosal blood flow with aggressive factors such as gastric acid

and pepsins (Boyd and Wormsley, 1985). Distortions of this balance can lead to the development of gastric disorders; collapse of protective factors or the overproduction of aggressive factors, or both, induced by stress, alcohol, or drugs can cause such distortions (Schwarz, 1910; Allen and Garner, 1980; Kauffman, 1981). Recently, colonization of *Helicobacter pylori* in the stomach has been shown to play an important role in various gastric disorders.

Gastric mucus is the most important protective factor of the gastric mucosa. It is produced by surface mucus cells and gland mucus cells in the stomach and secreted as mucin, which is a glycosylated protein that forms a gel layer on the gastric mucus membrane (Neutra and Forstner, 1987). The mucin production-associated genes mucin 5ac (*muc5ac*) and mucin 6 (*muc6*) encode mucin monomers, which are rod-shaped apomucin cores that are posttranslationally glycosylated.

Probiotics are defined by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO, 2001) as “live microorganisms, which when administered in adequate amounts confer a health benefit on the host.” Lactic acid bacteria and bifidobacteria are the most common types of microbes used as probiotics, and evidence is increasing that some probiotic lactobacilli offer beneficial health effects such as the suppression of harmful bacteria and metabolites in the gut, immune modulation, and mechanisms against anti-bowel disorders, anti-allergy, anti-inflammation, and anti-cancer (Isolaure et al., 2001; Reid and Hammond, 2005; Miyazaki and Matsuzaki, 2008; Cain and Karp, 2011). Bifidobacteria, which is used as a starter for various fermented milk products and dietary supplements, has proven probiotic effects (Shimakawa et al., 2003; Picard et al., 2005; Yakult Central Institute for Microbiological Research, 2012) and beneficial effects on the stomach. For example, Yamamoto et al. (1994) have reported that the administration of *Bifidobacterium bifidum* YIT 4007 reduces the severity of peptic ulcers and limits *H. pylori* infection in the gastric mucosa of affected patients. Nagaoka et al. (1994) found that, in rats, the cell wall polysaccharides of *B. bifidum* YIT 4007 mitigated the acute gastric injury induced by ethanol

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and acetic acid; moreover, fermented milk that contains *Bifidobacterium bifidum* BF-1 (**BF-1**) and *Streptococcus thermophilus* (**ST**) improved *H. pylori*-associated gastritis in a clinical trial, and BF-1 suppressed *H. pylori*-induced IL-8 secretion of human gastric cells (Miki et al., 2007) by inhibiting expression of *H. pylori*-induced genes and NF- κ B signaling pathway-associated genes (Shirasawa et al., 2010). However, the gastroprotective potential of BF-1 has yet to be fully elucidated in an animal model. Therefore, we used a rat model of acute gastric injury to investigate and compare the protective potential of living BF-1 with that of living ST.

MATERIALS AND METHODS

Bacteria and Culture

Bifidobacterium bifidum BF-1 (*B. bifidum* YIT 10347) and ST (*Strep. thermophilus* YIT 2021) were obtained from the Culture Collection Research Laboratory of the Yakult Central Institute for Microbiological Research (Tokyo, Japan). *Bifidobacterium bifidum* BF-1 was cultured in modified international lactobacilli Shirota (mILS) broth (Shimakawa et al., 2003) under anaerobic conditions using an AnaeroPack system (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan) for 20 h at 37°C.

Streptococcus thermophilus was grown in Difco lactobacilli de Man, Rogosa, and Sharpe (MRS) broth (Becton Dickinson Co., Sparks, MD) in static culture for 20 h at 37°C. Cells of each bacterial strain were collected by centrifugation at $3,000 \times g$ for 10 min at 4°C, washed twice in PBS, and resuspended in cold PBS. Before administration to rats, the number of colony-forming units in individual BF-1 or ST samples was determined by culturing samples on plates containing TOS propionate agar medium (Yakult Pharmaceutical Industry Co. Ltd., Tokyo, Japan) or Difco lactobacilli MRS agar (Becton Dickinson Co.).

Animals

All rats were maintained and treated in accordance with the guidelines of the Ethical Committee for Animal Experiments of the Yakult Central Institute for Microbiological Research. Male Sprague-Dawley rats [8 wk old, specific-pathogen-free (SPF) grade] were obtained from CLEA Japan Inc. (Tokyo, Japan) and were then housed in cages with wire mesh bottoms to prevent coprophagia. The cages were kept in a room with controlled lighting (lights on 0830 to 2030 h), temperature ($25 \pm 1^\circ\text{C}$), and humidity ($60 \pm 5\%$). Rats were given free access to a commercial nonpurified solid diet (MF diet; Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water.

Administration of Bacteria

After a 7-d adaptation period, rats ($n = 36$) weighing between 280 and 320 g were randomly divided into 3 groups. All rats were fasted for 20 h before the experiment, but had free access to water. Suspensions containing fresh BF-1 or fresh ST (each 7.5×10^{10} cfu/mL per rat) or vehicle only (PBS) were orally administered through a stainless tube attached to a 2.5-mL syringe.

Induction and Evaluation of Gastric Injury

To induce acute gastric injury, an aqueous solution containing 60% ethanol and 150 mM hydrochloride (1 mL/rat) was intragastrically instilled 2 h after administration of the bacterial suspension. For surgical collection of stomach samples, the rats were anesthetized under CO₂ 1 h after application of the ethanol-acid solution and then immediately killed for collecting samples. After the removal of gastric fluid, each stomach was inflated by injection of 10 mL of 2% formalin, and tissues in the inflated stomachs were fixed for 10 min. Thereafter, each stomach was opened along the line of greater curvature, washed in PBS, and spread on a board. To determine the total injury area, the length (mm) and width (mm) of the gastric lesions (areas of red streaks) were measured by an observer that was blinded to the treatment groups; the injury area was expressed as the lesion index. The percentage of injury was calculated using the following equation: lesion index of the test group/lesion index of the vehicle group (control group) $\times 100$. The drug benexate hydrochloride betadex (45 and 90 mg/rat; Shionogi & Co. Ltd., Osaka, Japan) was used as a positive control.

Analysis of mRNA Expression

Stomachs that had been obtained surgically were opened along the line of greater curvature and divided into right and left halves. After washing with PBS, tissue samples were collected from the fundic region in the right half of the stomach and immediately submerged in RNA-protective solution for 18 h at 4°C. Tissue samples were then homogenized in an acid guanidinium thiocyanate-phenol-chloroform mixture (ISOGEN; Nippon Gene Co. Ltd., Toyama, Japan), and total RNA was extracted from these homogenates. To analyze the expression of 7 genes (4 rat genes, 2 bacterial genes, and 1 rat house-keeping gene; Table 1), real-time reverse-transcription PCR was conducted using Power SYBR Green PCR master mix and a 7500 real-time PCR system (Applied Biosystems Inc., Foster City, CA). Rat PCR primers were designed with Primer Express software (Applied Biosystems Inc.); primer design was

Table 1. Target genes and primers used for real-time reverse-transcription PCR

Gene name ¹	Direction ²	5'–3' sequence	Reference
<i>muc5ac</i>	F	TCCAACCCTCACTACTGCTA	
	R	GACTTAGGACCAGCCTTTTG	
<i>muc6</i>	F	CATCACCTTTGTCTCCTACG	
	R	GAAGATGCTGTGGGTTTGGA	
<i>EGF</i>	F	CCGAGTCCTGTAGTAGTAAG	
	R	TTACTGCTCACGTTGCTGCT	
<i>bFGF</i>	F	CTGCTGGCTTCTAAGTGTGT	
	R	GTTATTGGACTCCAGGCGTT	
<i>Bifidobacterium bifidum</i>	F	TCAAGAAGGTGGTGAAGCAG	Matsuki et al. (2004)
	R	ACAACCTGGTCCTCAGTGTA	
<i>Streptococcus thermophilus</i>	F	GTGTGGATTGGTGGCTCTAT	Watanabe et al. (1998)
	R	GACTCATCGTACTCCTGCTT	
β -Actin	F	GTGTGGATTGGTGGCTCTAT	
	R	GACTCATCGTACTCCTGCTT	

¹*muc5ac* = mucin 5ac; *muc6* = mucin 6; *EGF* = epidermal growth factor; *bFGF* = basic fibroblast growth factor.

²F = forward; R = reverse.

based on the transcription sequence data of each gene obtained from PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>). Measurements from different RNA samples were normalized for RNA quantity relative to an internal control— β -actin, a house-keeping gene.

Evaluation of Gastric Mucin Production

The production of gastric mucin was evaluated using previously reported methods (Ishihara and Hotta, 1993). Briefly, gastric fluid containing soluble mucus was collected by washing a gastric fluid fraction from the left half of the stomach samples that had been prepared as described above as with PBS. The remaining left half of the stomach was immersed in 10 mL of PBS containing 2% (wt/vol) *N*-acetylcysteine for 5 min at room temperature to collect an adherent mucus gel layer fraction. Both fractions were combined; these mixtures were subjected to centrifugation at $10,000 \times g$ at 4°C for 10 min, and the supernatants were collected and defined as the gastric mucin fraction. As an index of gastric mucin production, the sugar content in the gastric mucin fraction was quantified by the phenol-sulfuric acid method; glucose was used as a standard for these measurements.

Statistical Analysis

Data are presented as means $\pm$ standard deviation. Statistical significance was determined using the Steel test or Dunnett test and $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects on Acute Gastric Injury

Preliminary experiments demonstrated that the oral administration of a positive control, benexate hydro-

chloride betadex, significantly inhibited the induction of injury; specifically, the positive control resulted in an approximate injury area that was 30% that of the vehicle administration group (data not shown). The effects of oral administration of living BF-1 were then examined in this rat model of acute gastric injury. Images of the stomachs of rats administered vehicle or BF-1, and then 2 h later treated with ethanol and acid to induce acute gastric injury, are shown (Figure 1). The rats given BF-1 exhibited relatively minor acute injury similar to a gastric ulcer in the corpus; these injuries were less severe than those of rats given vehicle. Figure 2 shows the rate of injury area in the rats given vehicle, BF-1, or ST (7.5×10^{10} cfu/rat). The rate of injury area was significantly lower in the BF-1 group ($48.9 \pm 43.2\%$) than in the vehicle group ($100 \pm 63.3\%$), which had a similar rate to the ST group ($89.0 \pm 52.4\%$). These findings indicate that the oral administration of living BF-1 has a gastroprotective potential that mitigated acute gastric injury in this rat model. Administration of BF-1 (7.5×10^{10} cfu/rat) 4 h before induction of gastric injury had significant gastroprotective potential, but administration of BF-1 (1.0×10^{10} cfu/rat) 2 h before gastric injury did not have significant effect (data not shown).

Effect on Stomach mRNA Expression

Next, to understand the effects of the administration of living BF-1 on the stomach, we analyzed the expression of several genes (Table 1) in the gastric corpus of rats. *Bifidobacterium bifidum* gene expression was observed in the BF-1 group ($5.6 \times 10^{-2} \pm 1.1 \times 0^{-1}/\beta$ -actin) and *Strep. thermophilus* gene expression was observed in the ST group ($4.7 \times 10^{-5} \pm 6.6 \times 10^{-5}/\beta$ -actin), but this bacterial expression was not observed in the other group. A gene associated with

Figure 1. Representative images of acute gastric injury in rats. Rats were given vehicle (PBS) or *Bifidobacterium bifidum* BF-1 (BF-1) and then treated with an aqueous solution of 60% ethanol with 150 mM hydrochloride (1 mL/rat) to induce acute gastric injury.

gastric protection (*muc5ac*) was expressed in rats that had been treated with BF-1, ST, or vehicle before the induction of acute injury (Figure 3). Expression of the *muc5ac* mRNA was significantly higher in the BF-1 group ($183.8 \pm 50.6\%$) than in the vehicle group ($100 \pm 21.2\%$), but no significant difference existed in *muc5ac* mRNA expression between the ST group ($122.2 \pm 28.2\%$) and the vehicle group or the BF-1 group. Expression of each of the other 3 genes monitored was not significantly affected by ST or BF-1 treatment (data not shown).

Effect on Gastric Mucin Production

We also assessed the effect of BF-1 and that of ST on the production of mucin derived from *muc5ac* expression in the stomach. The sugar content in the gastric mucin fraction was significantly higher in the BF-1 group ($129.74 \pm 32.2\%$) than in the vehicle control group ($100 \pm 17.4\%$), which had a similar content to that of the ST group ($86.6 \pm 23.2\%$). This finding indicates that oral administration of living BF-1 enhances mucin production in the rat stomach.

DISCUSSION

Reportedly, some probiotics have beneficial effects in the stomach in animal models and clinical trials. In particular, fermented milk containing BF-1 and ST reportedly alleviated *H. pylori*-associated gastritis in hu-

mans (Miki et al., 2007). However, the gastroprotective potential of BF-1 in acute gastric injury is unknown. Therefore, this study was designed to examine and compare the protective potential of living BF-1 and ST in an acute gastric injury rat model and to examine the underlying mechanisms.

Figure 2. Effect of oral administration of *Bifidobacterium bifidum* BF-1 (BF-1) or *Streptococcus thermophilus* (ST) on acute injury in ethanol-acid-treated rats. The administration of each sample and the induction of injury are described in Figure 1. The injury area was measured, and the percentages of injury area were calculated as follows: lesion index of the test group/lesion index of the vehicle (PBS) group (control group, Con) $\times 100$. Rats that had exogenous materials in the stomach samples were excluded from the analysis. Data are expressed as means $\pm$ SD. Con group: $n = 12$; BF-1 group: $n = 11$; ST group: $n = 12$. P -values were calculated using the Steel test. * $P < 0.05$.

Figure 3. Effect of oral administration of *Bifidobacterium bifidum* BF-1 (BF-1) or *Streptococcus thermophilus* (ST) on mucin 5ac (*muc5ac*) gene expression in the stomach of rats. The expression of target genes shown in Table 1 was analyzed by real-time reverse-transcription PCR. The ratio of *muc5ac* expression to β -actin expression was determined for each group and expressed as a percentage relative to the vehicle (PBS) group (Con). Rats that had exogenous materials in the stomach samples were excluded from the analysis. Data are expressed as means $\pm$ SD. Con group: $n = 12$; BF-1 group: $n = 10$; ST group: $n = 10$. P -values were calculated using the Dunnett test. ** $P < 0.01$.

Figure 4. Effect of oral administration of *Bifidobacterium bifidum* BF-1 (BF-1) or *Streptococcus thermophilus* (ST) on mucin production in rat stomach. Sugar content in the gastric mucin fraction prepared from rat stomachs was measured to evaluate mucin production in each group, and the data from the BF-1 and the ST groups were expressed as a percentage relative to the vehicle (PBS) group (Con). Rats that had exogenous materials in the stomach samples were excluded from the analysis. Data are expressed as means $\pm$ SD. Con group: $n = 12$; BF-1 group: $n = 8$; ST group: $n = 10$. P -values were calculated using the Dunnett test. * $P < 0.05$.

Oral administration of living BF-1 showed gastroprotective potential that mitigated acute gastric injury in this rat model, but administration of living ST did not (Figure 2). These findings support the concept that BF-1 is one, and possibly the only, component of fermented milk that is responsible for the mitigation of *H. pylori*-associated gastritis in humans. However, the gastroprotective potential of BF-1 at a dose of 7.5×10^{10} cfu/rat was such that the injury area of the BF-1 group was approximately 50% that of the vehicle group, and this protective potential was milder than that of benexate hydrochloride betadex at a dose of 45 mg/rat; benexate hydrochloride betadex resulted in an injury area that was approximately 30% that of the vehicle group.

Our findings also demonstrated that within 2 h after administration of living BF-1, but not living ST, mucin production was enhanced at both the mRNA level and the sugar content level in the stomach of rats (Figures 3 and 4). Reportedly, the cell wall polysaccharide of *B. bifidum* strain YIT 4007 has a similar gastroprotective potential in acetic acid and ethanol-treated rats; in these rats, it stimulates the production of epidermal growth factor and basic fibroblast growth factor in gastric tissue within 4 h after administration (Nagaoka et al., 1994). However, our study indicated that mRNA expression of epidermal growth factor and basic fibroblast growth factor was unchanged in the gastric corpus of the stomach 2 h after the administration of BF-1.

Mucin is a glycoprotein that forms the mucus gel layer that adheres to and protects the epithelial tis-

sue of the gastrointestinal tract. *Helicobacter pylori* infection reportedly causes the mucus gel layer in the stomach to become thinner (Newton, 2000); similarly, nonsteroidal antiinflammatory drugs decrease the magnitude of the mucus-bicarbonate barrier, disrupt the epithelial cell layer, reduce the surface hydrophobicity of epithelial cells, and diminish mucosal blood flow (Shorrock and Rees, 1989). Our findings indicate that the enhancement of mucin production following the oral administration of living BF-1 is an early response that plays an important role in protection against acute gastric injury in rats. In addition, BF-1 could have a gastroprotective function against the decrease of the mucus gel layer induced by *H. pylori*, nonsteroidal anti-inflammatory drugs, or other factors in humans.

It is known that diluted acetic acid such as vinegar acts as a mild irritant to the stomach, resulting in adaptive cytoprotection with an increase of gastric mucosal blood flow. Kawauchi et al. (2000) have reported that 0.3 to 1.0% acetic acid, but not 0.1% acetic acid, given acutely also reduce the severity of HCl/ethanol-induced gastric lesions. In our study, the concentration of acetic acid in BF-1 and ST cell suspension was 0.1%, and lactic acid in the suspension was less than the detection level (0.03%). This suggests that 0.1% acetic acid in the cell suspension could not influence the suppression of HCl/ethanol-induced gastric lesions in our study. Nagaoka et al. (1994) have reported that the cell wall polysaccharide of *B. bifidum* YIT 4007 had gastroprotective potential in acetic acid- and ethanol-treated rats. Therefore, it is suggested that mechanisms other

than those of lactic acid and acetic acid are associated with the gastroprotective potential of BF-1.

Based on in vitro assays, BF-1 adhered more tightly to gastric epithelial cells and to gastric mucus than did other strains of bifidobacteria and lactobacilli [A. Gomi, H. Shibahara-Sone, T. Iino (Yakult Central Institute for Microbiological Research, Tokyo, Japan), Y. Shimakawa (Yakult Central Institute for Microbiological Research), K. Miyazaki, and F. Ishikawa, unpublished data], indicating some interaction with the BF-1 on the gastric mucosa and gastric epithelial cells. Interestingly, we found that the BF-1 cells were 1,000 times more common than the ST cells in the stomach samples examined in this study. Moreover, BF-1 could tolerate gastric acid well, surviving for at least 4 h in the stomach of rats after oral administration (unpublished data). These characteristics may explain the observation that BF-1 enhances mucin production and offers subsequent gastroprotection. Alternatively, the possibility exists that BF-1 in the gut plays multiple roles in protection against acute gastric injury. More comprehensive studies are necessary to clarify the mechanism of the gastroprotective potential of BF-1.

CONCLUSIONS

We propose that BF-1 is a useful probiotic strain with the potential to confer gastroprotection and alleviate acute gastric injury by enhancing gastric mucin production. Further clinical trials of BF-1 treatments are necessary to determine whether such treatments could benefit human patients with gastric problems.

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