Alleviation of low-fiber diet-induced constipation by probiotic *Bifidobacterium bifidum* G9-1 is based on correction of gut microbiota dysbiosis

Yutaka MAKIZAKI[1], Ayako MAEDA[1], Yosuke OIKAWA[1], Saya TAMURA[1], Yoshiki TANAKA[1*], Shunji NAKAJIMA[1] and Hideki YAMAMURA[1]

[1]R&D Center, Biofermin Pharmaceutical Co., Ltd., Kobe, Hyogo, Japan

Constipation, a functional disorder of the digestive system, is common in children and adults and may compromise patient quality of life. Because many patients are not satisfied with the efficacy of existing therapies, in this study, we investigated the efficacy of the probiotic *Bifidobacterium bifidum* G9-1 (BBG9-1) in constipation induced by a low-fiber diet. After inducing constipation in rats by feeding a low-fiber diet, rats were fed a low-fiber diet mixed with BBG9-1 in 14 days to determine the efficacy of BBG9-1 for alleviating constipation. BBG9-1 significantly alleviated the dysbiosis induced by a low-fiber diet and improved the fecal counts, fecal weights, and fecal water contents. Moreover, it also improved organic acid concentrations in the cecal contents. These results suggested that in low-fiber diet-induced constipation, BBG9-1 could alleviate dysbiosis and constipation and may improve the intestinal environment, supporting its potential application in the treatment of constipation.
Key words: *Bifidobacterium bifidum* G9-1, functional constipation, probiotics, low-fiber diet

INTRODUCTION

Functional constipation is a gastrointestinal disorder without organic abnormalities that affects approximately 15% of the population in developed countries [1, 2]. Constipation can be classified into infrequent bowel motion or evacuation difficulties. Typical type of infrequent bowel motion is normal-transit constipation (NTC), in which the frequency and amount of defecation decrease despite normal fecal transport ability in the large intestine [3]. In NTC, due to a reduction in the amount of dietary fiber caused by excessive consumption of a westernized diet, the retention time of feces in the intestinal tract is extended, probably owing to insufficient accumulation of feces and absence of induction of peristaltic movement for defecation. As a result, the frequency of defecation decreases, fecal water absorption progresses, and the feces harden, thereby giving rise to symptoms of constipation, such as difficulty defecating. In addition to the resulting reduced quality of life in patients with constipation [4], sleep disturbances [5] and anal fissures [6] are also frequently observed in patients with constipation. Hence, alleviation of constipation is important for the maintenance of health and prevention of diseases. Treatments for constipation include enhanced intake of dietary fiber and use of laxatives. However, many patients with constipation are not satisfied with current treatment methods [7]; accordingly, options other than diet therapy and laxatives are being explored.

To date, many studies have suggested the involvement of intestinal flora in the development of various abdominal symptoms, and dysbiosis has been confirmed in patients with constipation [8, 9]. Constipation symptoms are observed in mice transplanted with fecal flora derived from patients with constipation, and intestinal flora have also been reported to be involved in the development of constipation [10]. Probiotics are live microorganisms that confer health benefits to the host when administered in appropriate amounts [11]. Indeed, probiotics have been reported to improve the intestinal flora in patients with various diseases [12] and alleviate symptoms of constipation [13]. However, the detailed effects of probiotics on dysbiosis in constipation have not been described.

The probiotic *Bifidobacterium bifidum* G9-1 (BBG9-1) has been shown to be useful for alleviating constipation in rats fed a low-fiber diet [14] and has been used as an intestinal medicine for several decades. Although the influence of BBG9-1 on the intestinal flora has been investigated, the analysis was carried out using culture methods, and detailed information, such as analysis by next-generation sequencing, has not been obtained.

Therefore, in this study, we aimed to elucidate the effectiveness of BBG9-1 in NTC and its influence on intestinal flora by next-generation sequencing using a constipation model that reflects constipation caused by insufficient dietary fiber.

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*Corresponding author. Yoshiki Tanaka (E-mail: tanaka_yoshiki@ biofermin.co.jp)

MATERIALS AND METHODS

Bacteria

Dried BBG9-1 (viable cell count: 1.94×10^{11} CFU/g) was used in this experiment. Dry bacterial powder was obtained from Biofermin Pharmaceutical (Kobe, Japan).

Animals

Six-week-old male Sprague Dawley rats were purchased from Japan SLC (Hamamatsu, Japan) and acclimated for 1 week before use in the experiment. Animals were housed individually in stainless steel 5-unit cages (CL-02036, W 755 × D 210 × H 170 mm; CLEA Japan, Inc., Tokyo, Japan) under specific pathogen-free conditions at room temperature (22 ± 3°C) and a humidity of 55% ± 15%, with a 12-hr light/dark cycle (07:00 to 19:00 hr). In addition, a CE-2 powdered diet (CLEA Japan) was given *ad libitum* as a standard diet, and tap water was freely available from a water supply bottle. All tests were conducted according to the animal experiment guidelines of Biofermin Pharmaceutical after approval by the animal experiment committee of Biofermin Pharmaceutical (approval number: 132-005).

Induction and evaluation of constipation

Constipation was induced by feeding a low-fiber diet based on standard feed (CE-2; Table 1) for 3 days. After induction of constipation, the control group was fed a diet containing 10% dextrin powder mixed with low-fiber diet feed, and the BBG9-1 group was fed a 10% BBG9-1 dry bacterial powder mixed with low-fiber diet feed. The normal group was fed CE-2 feed throughout the test period. Constipation was evaluated by measurement of fecal parameters (fecal number, weight, and water content). On day 14 of administration of BBG9-1, the cecum was removed under isoflurane anesthesia, and the contents of the cecum were rapidly frozen with liquid nitrogen and stored at −80°C until analysis.

Measurement of fecal number and weight

The number and weight of feces per day were measured between days 3 and 14 using all feces excreted during each 24-hr period, with 10:00 AM as the starting time point every day.

Measurement of fecal water content

Fecal water content was determined by collecting fresh feces discharged by slightly compressing the abdomen. After measuring the weight of the feces, samples were dried at 90°C for 24 hr, and the moisture content was calculated using the weight change before and after drying.

Measurement of the pH of cecal contents

The pH of the cecal content, suspended in 9× ion-exchanged water, was measured using a pH meter (D-54, Horiba, Kyoto, Japan).

Table 1. Contents of the standard diet and low-fiber diet

Ingredients	Contests (%)	
	CE-2 (standard diet)	Low-fiber diet
Moisture	8.89	9.00
Crude protein	24.88	24.90
Crude fat	5.03	3.90
Crude fiber	4.63	0.90
Crude ash	6.79	6.00
Nitrogen-free extract	49.78	55.30

Measurement of organic acid concentrations in cecal contents

The concentrations of organic acids in the cecal contents were measured according to the method of Nagano *et al.* [15].

Microbiological analyses of cecal contents

Comprehensive 16S rRNA sequence analysis was performed for studying the microbiota. DNA was extracted from cecal content using the bead-phenol method. Sequence analysis of the V3–V4 region of the 16S rRNA gene was performed using the MiSeq platform according to the method of Fadrosh *et al.* [16]. Next, analysis of the sequence read data obtained from MiSeq was performed using the QIIME pipeline [17]. Reads were merged using fastq-join, and quality filtering (QV ≥ 25) was performed using USEARCH v 6.1. The read data that passed the filtering and chimeric read check were used for flora analysis. We randomly extracted 5,000 reads per specimen and created an operational taxonomic unit (OTU) with 97% homology as the threshold value using USEAECH. A homology search was performed for the representative sequence of the OTU using UCLUST, and identification up to the order level of each read was performed.

Calculation of the dissimilarity (Bray-Curtis distance) of the intestinal microflora composition of each sample was performed using the vegdist function (vegan package) of the statistical analysis software R. Based on the obtained value, principl coordinate analysis (PCoA) was performed using the dsv.pco function.

Statistical analysis

The experimental results obtained were expressed as means ± standard errors. We conducted a test for normality in each group and tested the variance using Bartlett's test if a normal distribution was observed. In cases of equal variance, data were tested using Tukey-Kramer tests. If variances were unequally distributed or if a normal distribution was not observed, data were tested using Steel-Dwass tests. Results with p values of less than 0.05 were considered significant.

RESULTS

Effects of BBG9-1 on stool parameters

In the control group, there was a significant decrease in the number and weight of feces from day 1 compared with
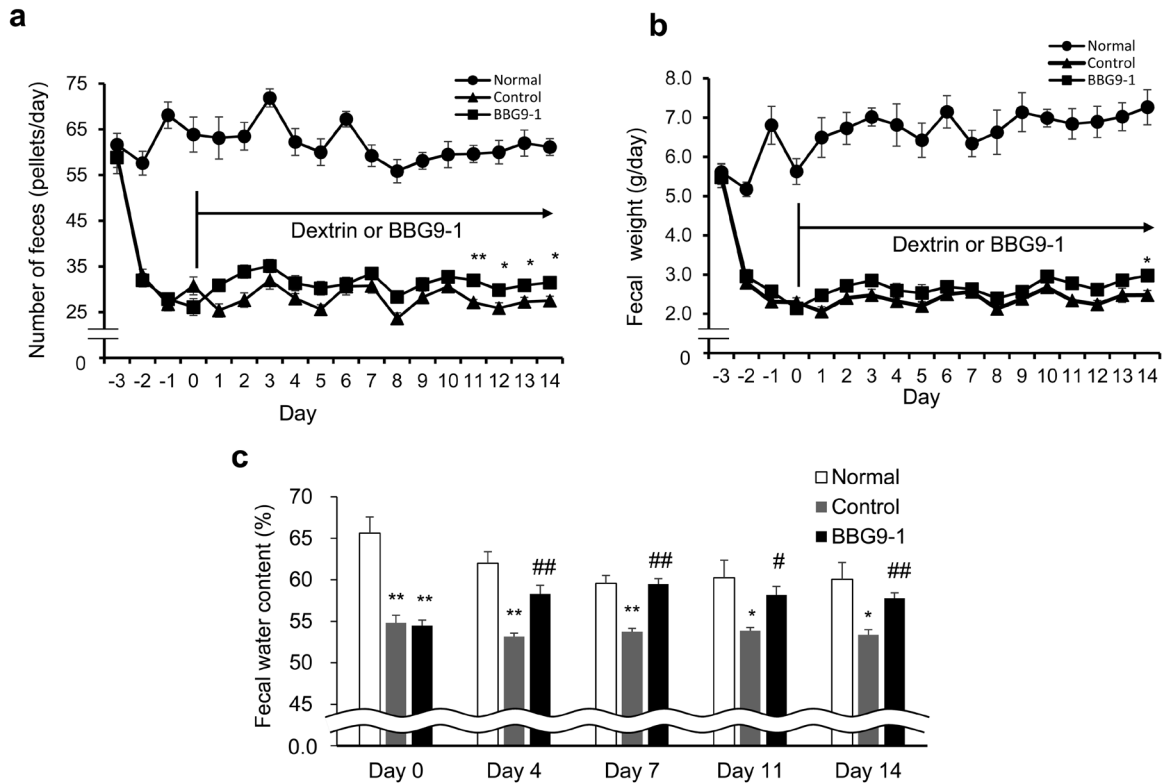
Fig. 1.   Effect of BBG9-1 on low-fiber diet-induced constipation.

a) Number of feces and b) fecal weight were measured during low-fiber diet administration. c) Fecal water content was measured in each group at days 0, 4, 7, 11, and 14. BBG9-1 was administered after induction of constipation by feeding a low-fiber diet for 3 days (day 0). Values are means ± SE of 8 animals. *p<0.05 vs. normal; **p<0.01 vs. normal; #p<0.05 vs. control; ##p<0.01 vs. control.

those in the normal group. In the BBG9-1 group, there was a significant increase in the number of feces on days 11–14 (Fig. 1a) and a significant increase in fecal weight on day 14 (Fig. 1b) compared with those in the control group. Furthermore, the fecal water content in the BBG9-1 group was significantly increased compared with that in the control group on all days (Fig. 1c).

Effects of BBG9-1 on organic acids and pH in cecal contents

The intestinal butyric acid contents in the control and BBG9-1 groups were significantly lower than those in the normal group; however, there was a significant increase in the intestinal butyric acid content in the BBG9-1 group compared with that in the control group. Lactic acid was found to be significantly lower in the control group than in the normal group. There were no significant differences in lactic acid in the BBG9-1 and control groups. Significant increases were observed in valeric acid contents in the control and BBG9-1 groups compared with that in the normal group, and a significant increase in succinic acid content was observed in the BBG9-1 group compared with that in the normal group. There were no changes in acetic acid contents in any group. There were no significant differences in pH between the

BBG9-1 and control groups (Table 2).

Effects of BBG9-1 on the composition of cecal microbiota

The control group showed a flora profile that differed from that of the normal group, although the BBG9-1 group showed the same profile as the normal group, with similar bacterial flora (Fig. 2a).

A significant decrease in *Clostridiales* was observed in the control group compared with that in the normal group, and significant increases in *Bacteroidales*, *Erysipelotrichales*, *Selenomonadales*, and *Verrucomicrobiales* were also observed. In contrast, the bacterial changes that occurred in the control group were not observed in the BBG9-1 group (Fig. 3).

DISCUSSION

In this study, we evaluated constipation caused by a lack of dietary fiber intake in rats and aimed to understand the effects of BBG9-1 on low fiber-induced constipation in rats through determination of its influence on the internal flora and metabolites.

NTC occurs due to decreased insoluble matter, insufficient formation of feces, and decreased frequency of defecation. As the resident time in the intestinal tract increases, fecal water

Table 2. Effect of BBG9-1 on organic acid and pH in cecal contents of low-fiber diet-induced constipation model rats

Treatment	Succinic acid (mg/g)	Lactic acid (mg/g)	Acetic acid (mg/g)	Propionic acid (mg/g)	n-Butyric acid (mg/g)	n-Valeric acid (mg/g)	pH
Normal	0.05 ± 0.01	0.54 ± 0.19	3.64 ± 0.12	1.17 ± 0.05	3.65 ± 0.15	0.13 ± 0.004	6.47 ± 0.07
Control	0.07 ± 0.01	0.15 ± 0.02*	3.82 ± 0.21	1.71 ± 0.06**	0.72 ± 0.04**	0.17 ± 0.01**	6.95 ± 0.01**
BBG9-1	0.14 ± 0.01**	0.21 ± 0.05	3.68 ± 0.27	1.53 ± 0.05**	1.16 ± 0.14**,##	0.18 ± 0.01**	6.79 ± 0.04 **

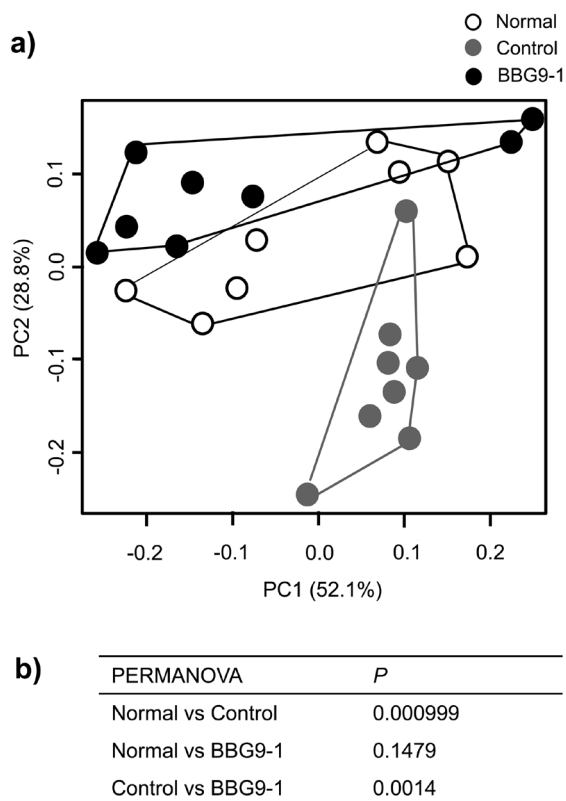Values are means ± SE of 8 animals. *$p<0.05$ vs. normal; **$p<0.01$ vs. normal; ##$p<0.01$ vs. control.

Fig. 2. Effect of BBG9-1 on the structure of cecal microbiota in rat models of low-fiber diet-induced constipation. a) Principal coordinates analysis (PCoA) showed the clustered communities of cecal microbiota, based on the Bray-Curtis dissimilarity between samples. b) Results of permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis distance in cecal microbiota. The test was done using 1,000 permutations.

is mostly absorbed, leading to hardening of the feces and difficulties with defecation. In our current model, significant decreases in the weight and number of feces were observed from day 1 of feeding the low-fiber diet; the values reached a plateau on day 3. Because fecal water content decreased significantly by day 3, this model seemed to reflect NTC on day 3 of the low-fiber diet. Thus, it was considered a useful model for evaluating NTC. When BBG9-1 was administered to this constipation-induced model, the amount and number of defecations and the fecal water content increased, suggesting that BBG9-1 was effective as a treatment for NTC.

Alleviation of dysbiosis and constipation by administration of BBG9-1 in this model suggested that alleviation of constipation by BBG9-1 treatment may be related to the effects of the probiotic on dysbiosis. Administration of BBG9-1 increased *Clostridiales*, the order of bacteria to which the butyric acid-producing bacteria (*Roseburia*, *Eubacterium*, and *Anaerostipes*) belong, and increased butyric acid, which activates colonic movement [18–20]. However, BBG9-1 did not have the ability to produce butyrate directly. Therefore, BBG9-1 may have alleviated constipation by increasing butyric acid-producing bacteria, alleviating dysbiosis, and subsequently increasing butyric acid. In addition, BBG9-1 produces lactic acid and acetic acid, although no significant increase in either was observed in this study. Butyric acid-producing bacteria have been reported to assimilate acetic acid to produce butyric acid [21], and bacteria that assimilate lactic acid to produce butyric acid have also been reported [20, 22]. Therefore, in this study, the lack of increases in lactic acid and acetic acid may be a result of butyric acid-producing bacteria actively utilizing the lactic acid and acetic acid produced by BBG9-1. In a preliminary study, BBG9-1 was confirmed to produce higher amounts of lactic acid and acetic acid than the type strain of *B. bifidum* (data not shown). Therefore, correction of gut microbiota dysbiosis may be related to the capacity to produce short-chain fatty acids.

BBG9-1 has been used as an intestinal medicine for several decades, and the efficacy of a 10% BBG9-1-containing diet for constipation in rats fed a low-fiber diet was confirmed previously [14]. Therefore, in this study, we used a 10% BBG9-1-containing diet to elucidate the mechanisms through which BBG9-1 alleviated constipation. Although experimental animal models are useful for evaluating the potential efficacy of drugs for disease treatment, the results of animal model studies may not directly reflect dose setting for human patients. Further studies are needed to determine the relationship between BBG9-1 dosage and constipation.

In conclusion, our findings in this study suggested that improvement of the intestinal environment, as represented by alleviation of dysbiosis and an associated increase in butyric acid, may contribute to the therapeutic effects of BBG9-1 on NTC. Therefore, the probiotic BBG9-1 may have applications in the treatment of constipation.
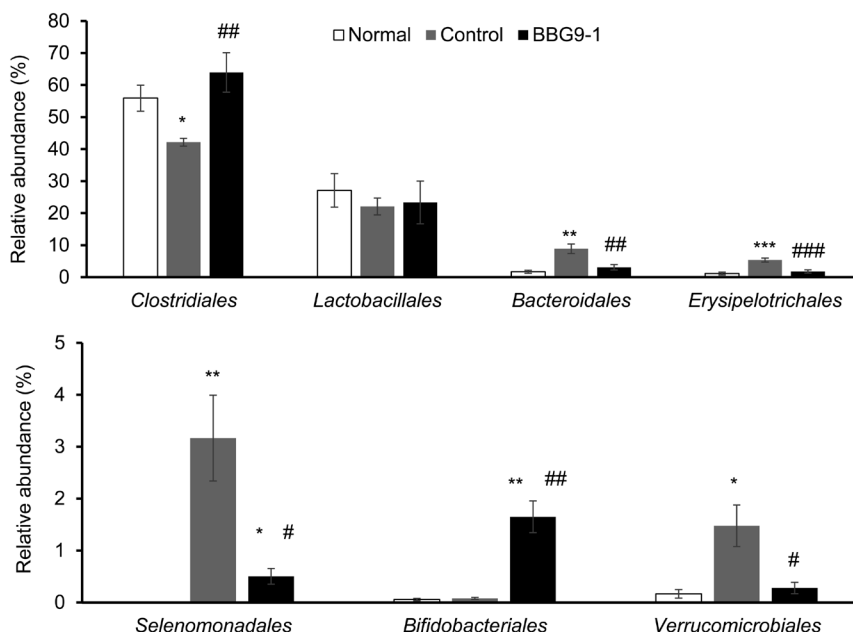
Fig. 3. Effect of BBG9-1 on cecal microbiota of low-fiber diet-induced constipation models. Relative abundance of each bacterial order was analyzed by next-generation sequencing of bacterial 16S rDNA. Each value is presented as the mean ± SE of 8 animals. *p<0.05 vs. normal; **p<0.01 vs. normal; #p<0.05 vs. control; ##p<0.01 vs. control; ###p<0.001 vs. control.

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