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本实验调查了膳食补充双歧杆菌发酵大豆下胚轴（BFSH）对老年人大肠菌群组成的影响。每日给予14名老年志愿者4g含有109 cfu / g 双歧杆菌的BFSH，连续摄取10天，然后是暂停摄取10天，该周期重复50天。 肠道微生物（拟杆菌属、双歧杆菌、乳酸杆菌、大肠杆菌、产气荚膜梭菌）检测显示给予BFSH导致双歧杆菌明显增多和类杆菌减少。评估的排便频率，PH值和水分含量无显著性差

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益生菌对湿疹或特应性皮炎的预防作用存在争议。我们调查研究了补充益生菌是否可以预防高风险的婴儿湿疹的发生。在一个随机，双盲，安慰剂对照试验中，112名有过敏性疾病家族史的孕妇分别摄取两歧双歧杆菌BGN4， 乳双歧杆菌AD011和嗜酸乳杆菌AD031的复合菌粉和安慰剂，从产前4~8周摄取到分娩后6个月，前3个月纯母乳喂养，4~6个月母乳喂养或者奶粉喂养。对婴儿的临床症状检测到1岁，测量对常见食物过敏原总的和特异性lgE。共有68名婴儿完成了该临床研究。益生菌组在1岁时湿疹的发病率明显低于安慰剂组（18.2% VS 40.0% P=0.048)，另外在12个月湿疹的累计发病率明显降低（36.4% VS 62.9%, p=0.029). 两组间的血清总lgE水平及食物致敏性无显著性差异。产前和产后补充两歧双歧杆菌BGN4，乳双歧杆菌AD011,嗜酸乳杆菌AD031复合益生菌是一个有效预防婴幼儿第一年高风险湿疹的方法。

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临床菌株：长双歧杆菌BORI和嗜酸乳杆菌AD031

本研究中共有57名轮状病毒感染住院婴幼儿。将患儿随机分成两组1. 摄取长双歧杆菌BORI，嗜酸乳杆菌AD031(N=28）一共三天。2 安慰剂组：不含益生菌的脱脂牛奶 N=29）同时执行标准的腹泻治疗方法。两组患儿的年龄，性别，血液特征均无差异。益生菌组腹泻持续时间（4.38±1.29， N=28)明显短于安慰剂组（5.61±1.23，N=29）p值为0.001。益生菌治疗可以改善发热时间（P=0.119），腹泻次数（p=0.119)，呕吐次数（p=0.331)等症状，但两者间差异无统计学意义。两组患者均无严重不良事件发生，不良事件发生频率无差异。

肠易激综合征的临床研究（成年人）

益生菌对韩国成年人肠易激综合征的影响

Effect of Probiotics on Symptoms in Korean Adults with Irritable Bowel Syndrome with Rotavirus Infection
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研究背景/目的：肠易激综合征是一种棘手的疾病，一些益生菌据说具有显著的免疫调节作用，因此，我们设计了一项随机，双盲，安慰剂对照的试验，以评估对韩国成年人IBS的影响。 方法：随机选取复合Rome III标准的肠易激综合征患者，分别给与复合益生菌和安慰剂治疗。总菌数为200亿，每天2次，一共摄取8周。主要的结果变量症状评分包括腹部疼痛，胀气，排便不适和症状总分。采用视觉模拟量表量化病情严重程度。次要结果变量包装生活质量和排便习惯，包括排便频率和粪便形态。 结果 ：36例和34例患者患者分别随机分为益生菌组和安慰剂组，意向性治疗结果显示8周后疼痛明显减轻，益生菌组和安慰剂组分别是-31.9和-17.7（P=0.045)。 在58例患者中，腹痛，排便不适及总分减少的更为显著，而且在大便量表的得分至少为3分。结论：含两歧双歧杆菌BGN4、嗜酸乳杆菌AD031等菌种的复合益生菌是安全有效的，尤其适用于排泄正常或稀便的患者。

腹泻型肠易激综合征的临床研究（成年人）

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临床菌株：长双歧杆菌BORI，两歧双歧杆菌BGN4， 乳双歧杆菌AD011，婴儿双歧杆菌IBS007和嗜酸乳杆菌AD031

目前有很多的研究显示益生菌对腹泻型肠易激综合征有效，然而其作用机制还有待于阐明。在这个研究中，为了了解益生菌在IBS-D中作用的代谢组学的特征，比较多种益生菌（一个乳杆菌，四个双歧杆菌）N=32和安慰剂组 n=31的IBS症状变化和受试者尿液样本的代谢组学特征。通过8天的摄取（3次/天），不满意的排便习惯以及排便频率明显得到改善。而且，益生菌组明显变化了七个代谢物包括十六烷酸甲酯（PAME), 4,6-二羟基喹啉和DOBA。根据IBS病情尺度调查表和IBS生活质量调查问卷，IBS-SSS应答者显示更好的PAME水平，IBS-QoL应答者显示更高的DOBA水平。这表明这些代谢物可能作为潜在的生物标志物来预测益生菌对腹泻型肠易激综合征的功效。

改善认知功能和抑郁情绪的临床研究（老年人）

益生菌补充剂通过肠道菌群的变化改善老年人的认知能力和情绪，随机，双盲，安慰剂对照，多中心试验

Probiotic Supplementation Improves Cognitive Function and Mood with Changes in Gut Microbiota in Community-Dwelling Older Adults: A Randomized, Double-Blind, Placebo-Controlled, Multicenter Trial
临床菌株：长双歧杆菌BORI 和 两歧双歧杆菌BGN4

益生菌在患者以及动物模型中已被证实通过脑肠轴改善认知障碍和抑郁情绪。然后，益生菌在健康老年人大脑功能中的有益作用还不清楚。因此我们进行了一项随机，双盲，安慰剂对照的多中心试验，以确定益生菌对社区居住的老年人认知能力和情绪的影响。63名老年人服用安慰剂和含有两歧双歧杆菌BGN4和长双歧杆菌BORI的益生菌12周。采用16s RNA测序和生物学信息分析肠道微生物群。研究人员利用大学协作建立了阿尔茨海默病，生活满意度量表，压力问卷，老年抑郁量表，积极情绪和抑郁情绪量表的注册表对大脑功能进行了测定。用酶联免疫吸附法测定血脑源性神经营养因子（BDNF). 在第12周，益生菌组中引起炎症的肠道细菌的相对丰度显著降低（P<0.05), 益生菌组在心理灵活性和压力评分方面比安慰剂组有更好的改善（p<0.05)。与安慰剂相比，益生菌组显著提高了血清的BDNF水平（p<0.05)。值得注意的是，益生菌显著转移的肠道微生物（真杆菌和梭状芽胞杆菌）与血清BDNF水平呈显著负相关。综上所述，益生菌促进健康老年人的精神灵活性并缓解压力，同时也引起肠道菌群的变化。这些结果为益生菌作为老年人健康饮食的一部分，具有促进健康的特性提供了证据。

溃疡性结肠炎的临床研究

益生菌治疗溃疡性结肠炎的长期效应：一项临床研究

The long-term effects of probiotics in the therapy of ulcerative colitis: A clinical study
临床菌株：两歧双歧杆菌BGN4

目的：肠道失调似乎是炎症性肠病的主要原因，益生菌似乎是对对抗炎症性肠病发生提供了适当的支持。事实上，益生菌混合物和抗炎症药物是对抗炎症性肠病的武器。本研究评估联合治疗（美沙拉嗪和唾液乳杆菌，嗜酸乳杆菌和两歧双歧杆菌BGN4)对溃疡性结肠炎的长期效应（2年）。方法 ：纳入60例中至重度溃疡性结肠炎患者，其中30例每日口服美沙拉嗪1200mg，30名患者每天口服美沙拉嗪1200mg每天两次口服唾液乳杆菌，嗜酸乳杆菌和两歧双歧杆菌BGN4益生菌混合物。治疗持续两年，根据改良的的Mayo疾病活动度指数进行临床反应评价。结果。所有接受联合治疗的患者都比对照组有更好的改善。特别是益生菌的有益作用，即使在治疗两年后也很明显。结论。抗炎药物和益生菌的长期治疗方式是可行的，可以在轻度至中度溃疡性结肠炎中替代皮质类固醇。

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RESEARCH NOTE

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Effect of *Bifidobacterium*-fermented Soy Hypocotyls Intake on the Composition of Human Large Intestinal Bacteria in the Elderly

Nam Ju Kim, Se Jin Park, Eun Mi Yum, Hye Young Kim, Sun Ho Lee[1], Ji Hoon Min[1], Myung Soo Park[1] and Geun Eog Ji[1]*

Department of Food and Nutrition, Seoul National University, Seoul 151-742, Korea

[1]Research Center, Bifido Inc., Seoul 151-742, Korea

Abstract Effect of dietary supplementation of *Bifidobacterium*-fermented soy hypocotyls (BFSH) on the composition of large intestinal bacteria of the elderly people was investigated. Four grams of BFSH containing 10^9 cfu/g *Bifidobacterium* were administered daily to 14 elderly volunteers every day for 10 days, followed by 10 days of non-intake period, and the cycle was repeated for 50 days. Composition of the intestinal bacteria (*Bacteroides, Bifidobacterium, Lactobacillus, E. coli, Clostridium perfringens*) examined revealed that administration of BFSH resulted in a marked increase in *Bifidobacterium* and a decrease in *Bacteroides*. Stool evacuation frequencies, pH, and water contents of the fecal samples did not change significantly.

Keywords: *Bifidobacterium*, soy, intestinal bacteria, elderly

Introduction

The human intestinal tract contains a complex and dynamic bacterial population, whose cell numbers amount to 10^{14} cfu (colony forming units) per gram (1). The metabolic activities of the bacteria can exert both harmful or beneficial effects on the human host depending on the species, diet, and other various environmental factors (1). The number of putrefactive bacteria such as *Clostridium perfringens* are known to increase with the aging of the human host, leading to increased production of ammonia, H_2S, and amines, which can aggravate the intestinal environment of the elderly people (2). On the other hand, the number of beneficial bacteria such as *Bifidobacterium* decreases at a significant level during aging. Upon examining the composition of the intestinal bacteria of the Koreans, Ji reported that number of *Bifidobacterium* was lower and that of *Cl. perfringens* higher in the elderly people (above 65 years group) than the young people (3). In spite of the recent marked increase in elderly people in Korea, little studies have been conducted to improve the health state in terms of the intestinal bacterial balance for the elderly people. Probiotics are well known to affect the composition of indigenous microflora and may have several beneficial effects on the human health such as the maintenance of a balanced flora, alleviation of lactose intolerance symptom, and resistance to enteric pathogens (4). Modes of action of probiotics are suggested to be the colonization of the gastro-intestinal tract, prevention of pathogen overgrowth, neutralization of enterotoxins, modulation of the activity of bacterial enzymes in the large intestine, improvement of the digestive capacity of the small intestine, and adjuvant effect on the immune system (5). In most cases, clinical studies were preceded by laboratory-based research, utilizing animal models or *in vitro* culture systems, to provide preliminary evidence on the intestinal condition. In this study, soybean hypocotyls, part of the axis of soybean embryo below the cotyledons, were fermented with *Bifidobacterium*. The effect of dietary supplementation of *Bifidobacterium*-fermented soy hypocotyls (BFSH) on the intestinal bacteria of the elderly people was investigated.

Materials and Methods

Soybean hypocotyls were ground and suspended in 10 volumes of water. The soy hypocotyls medium was flushed with gas mixture (95% N_2 and 5% CO_2) to remove dissolved oxygen. For fermentation, *Bifidobacterium* sp. BGN4 previously characterized (6) was used. After autoclaving, fresh *Bifidobacterium* sp. BGN4 cultures were inoculated at a 1:50 ratio. The suspension fermented for 24 hr was lyophilized until dryness. It contained *Bifidobacterium* counts of 10^9cfu/g dry weight. Four grams (two grams after breakfast and two grams after dinner) of the powder were administered to 14 elderly volunteers (7 each males and females) every day for 10 days, followed by 10 days of non-intake period. The nonintake-intake cycle was repeated for 50 days. During the study period, all subjects were asked to avoid consumption of fermented food and other probiotic products. The composition of intestinal bacteria was examined at the last day of each period using fresh fecal samples. The serially diluted samples were plated on various selective and non-selective media as described by Ji (3). For counting anaerobic organism, Anoxomat (MART, Netherland) was used to equilibrate

*Corresponding author: Department of Food and Nutrition, Seoul National University, Seoul 151-742, Korea
Tel: 82-2-880-8749
Fax: 82-2-884-0305
E-mail: geji@bifido.com

Table 1. Effect of BFSH intake on the composition of human feces

	Non-intake / Intake cycle				
	Before intake	First intake	First non-intake	Second intake	Second non-intake
Microorganism	(Log of CFU per gram wet feces)				
Bifidobacterium	9.0±0.5	9.5±0.5	9.0±0.8	10.0±0.4	9.1±0.8
Bacteroides	9.6±0.4	8.7±1.5	10.1±0.4	9.7±0.3	9.9±0.6
Lactobacillus	ND	7.2±1.4	7.5±1.3	8.0±1.4	7.5±1.2
Clostridium	4.5±0.7	4.8±1.3	5.1±0.6	4.7±0.9	5.3±1.1
E. coli	8.6±0.5	7.6±1.0	7.8±1.0	7.9±2.3	7.6±0.8
Total aerobic bacteria	8.8±0.4	7.5±1.0	8.1±0.8	8.4±0.6	8.4±0.5
Water content (%)	73.6±8.6	70.2±9.1	70.2±8.5	71.7±9.2	73.2±8.9
pH	6.9±0.6	6.8±0.8	6.8±0.9	6.9±1.0	6.5±0.6

All numbers in Mean±S.D. ND means not determined.

the anaerobic chamber and the plate was incubated 2-3 days at 37°C. The pH was measured by a pH meter (Fisher Model 10, USA) after the suspension of fecal samples with 4 volumes of water. The moisture contents were analyzed after drying at 105°C in a drying oven. Group means comparisons were tested for significance by Student's *t*-test. Statistical significance was defined as $P<0.05$. Fecal composition results of the subjects are expressed as Means±S.D..

Results and Discussion

Bioconversion of soy oligosaccharides and isoflavones, and their corresponding enzymes, alpha-galactosidase and beta-glucosidase, has been previously characterized in our laboratory during soy fermentation using *Bifidobacterium* (7, 8). For the present study, we used a hypocotyl portion of the soy for fermentation. The effect of dietary supplementation with BFSH on intestinal bacteria of the elderly people is shown in Table1.

Among the examined bacteria, viable *Bifidobacterium* cell number showed the most pronounced increase during intake, whereas that of *Bacteroides* decreased following the consumption of BFSH. No significant changes were obtained for *E, coli, Lactobacillus,* and *Cl. perfringens*. *Bifidobacterium* comprising more than 90% of the intestinal bacteria in breast-fed infants decreases down to about 10% after weaning and during the following life time period (1). *Bifidobacterium* has been considered to confer various beneficial effects on the human hosts, whereas *Bacteroides* are known to produce various putrefactive compounds and are the most frequently found anaerobic infectious agents in various tissues aside from the intestine (1, 2). In this context, our result may be interpreted as an improvement in the balance of the intestinal bacteria by the supplementation of BFSH. Further analysis needs to be performed on whether the observed effects were solely due to administered *Bifidobacterium* or whether some other components of BFSH are responsible. The effect of BFSH intake on pH, moisture contents, and stool evacuation frequencies were not eminent. Throughout the experiment, the participants showed a high interest in the experiments and willingness to donate their fecal samples. More research on the development of the probiotic foods for the elderly is warranted considering that the population of the elderly will soon double by year 2020. Further studies should delineate the mechanism of the probiotic action, the evaluation of the physiological effect, and the efficient delivery method of probiotic products for special targets (e.g., age group, intestinal organ, etc.) in more detail.

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PEDIATRIC ALLERGY AND IMMUNOLOGY

Effect of probiotic mix (*Bifidobacterium bifidum, Bifidobacterium lactis, Lactobacillus acidophilus*) in the primary prevention of eczema: a double-blind, randomized, placebo-controlled trial

Kim JY, Kwon JH, Ahn SH, Lee SI, Han YS, Choi YO, Lee SY, Ahn KM, Ji GE. Effect of probiotic mix (*Bifidobacterium bifidum, Bifidobacterium lactis, Lactobacillus acidophilus*) in the primary prevention of eczema: a double-blind, randomized, placebo-controlled trial.
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Controversy exists regarding the preventive effect of probiotics on the development of eczema or atopic dermatitis. We investigated whether supplementation of probiotics prevents the development of eczema in infants at high risk. In a randomized, double-blind, placebo-controlled trial, 112 pregnant women with a family history of allergic diseases received a once-daily supplement, either a mixture of *Bifidobacterium bifidum* BGN4, *B. lactis* AD011, and *Lactobacillus acidophilus* AD031, or placebo, starting at 4–8 wks before delivery and continuing until 6 months after delivery. Infants were exclusively breast-fed during the first 3 months, and were subsequently fed with breastmilk or cow's milk formula from 4 to 6 months of age. Clinical symptoms of the infants were monitored until 1 yr of age, when the total and specific IgE against common food allergens were measured. A total of 68 infants completed the study. The prevalence of eczema at 1 yr in the probiotic group was significantly lower than in the placebo group (18.2% vs. 40.0%, $p = 0.048$). The cumulative incidence of eczema during the first 12 months was reduced significantly in probiotic group (36.4% vs. 62.9%, $p = 0.029$); however, there was no difference in serum total IgE level or the sensitization against food allergens between the two groups. Prenatal and postnatal supplementation with a mixture of *B. bifidum* BGN4, *B. lactis* AD011, and *L. acidophilus* AD031 is an effective approach in preventing the development of eczema in infants at high risk of allergy during the first year of life.

Ji Yeun Kim[1], Jung Hyun Kwon[2], So Hyun Ahn[2], Sang Il Lee[2], Young Shin Han[3], Young Ok Choi[1], Soo Young Lee[4], Kang Mo Ahn[2]* and Geun Eog Ji[1,5]*

[1]Department of Food and Nutrition, College of Human Ecology, Seoul National University, San 56-1, Shinlimdong, Kwanakku, Seoul, Korea, [2]Department of Pediatrics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Cheoncheondong, Jangangu, Suwon, Gyeonggido, Korea, [3]Department of Pediatrics, Samsung Medical Center, 50 Irwon-dong, Gangnam-gu, Seoul, Korea, [4]Department of Pediatrics, Ajou University School of Medicine, San 5, Woncheondong, Youngtonggu, Suwon, Gyeonggido, Korea, [5]Research Institute, Bifido Inc. 688-1, Hongcheongun, Kangwondo, Korea

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Geun Eog Ji, Department of Food and Nutrition, College of Human Ecology, Seoul National University, San 56-1, Shinlimdong, Kwanakku, Seoul 152-742, Korea
Tel.: +82 2 880 8749, +82 11 729 8672
Fax: +82 2 884 0305
E-mail: geji@snu.ac.kr
*They contributed to correspondence authors equally to this work.

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Eczema or atopic dermatitis is a common chronic inflammatory skin disease, mostly occurring in children (1). A recent study has shown that the worldwide prevalence of eczema in childhood was increasing (2), and the prevalence of eczema in Korean children aged 6–12 yrs increased between 1995 (19.7%) and 2000 (27.5%) (3). It is speculated that the increasing prevalence of allergic diseases in developed countries is associated with the so-called 'hygiene hypothesis', in which a lack of infections or other microbial exposures at an early age leads to Th2-dominant immune status and the subsequent development of allergic diseases (4, 5).

Recently, the role of intestinal microflora has been emphasized in the maintenance of normal gut barrier function and development of tolerogenic immune status (6). Mice raised in a germ-free environment failed to develop oral tolerance and had a persistent Th2-dependent immune response, while reconstitution of intestinal microbes during the neonatal period could reverse this immune deviation (7). Infants with allergic diseases showed less intestinal colonization by *Lactobacillus* or *Bifidobacterium* and more colonization by *Clostridium* relative to non-allergic infants (8–10). It is also known that specific intestinal bacteria bring about immune tolerance via the up-regulation of inhibitory Toll-interacting protein (11). These findings suggest that certain gut microbes modulate regulatory T cells, leading to the suppression of allergic disorders (12).

It remains a matter of controversy whether the modification of intestinal microflora by supplementation with probiotic bacteria in early life is effective in preventing eczema. Prenatal and postnatal supplementation with *Lactobacillus rhamnosus* GG has been shown to reduce the prevalence of atopic eczema at 2 yrs of age. This protective effect even continued during 7 yrs of follow-up (13, 14); however, it did not affect the sensitization rate or the development of asthma or allergic rhinitis. In another study, early supplementation with *Lactobacillus acidophilus* (LAVRI-A1) did not reduce the prevalence and severity of atopic dermatitis, but resulted in an increased proportion of infants with allergic sensitization at 1 yr of age (15). Those contradictory findings imply that further studies are needed to assess whether supplementation with probiotic bacteria at an early age has a preventive effect on eczema.

In the present study, we investigated whether prenatal and postnatal administration of a mixture of *Bifidobacteria* and *Lactobacillus* could prevent the development of eczema and sensitization against common food allergens in infants at high risk of atopic disease.

Materials and methods

Study design and participants

This study was a randomized, double-blind, placebo-controlled trial designed to evaluate the preventive effect of probiotics on the development of eczema. A total of 112 pregnant women with a family history of allergic diseases were recruited at Samsung Medical Center (Irwon-dong, Gangnam-gu, Seoul, Korea) from January 2005 through January 2006. A family history of allergic diseases was defined according to the following criteria: (i) when at least one parent or older sibling of the fetus had eczema, as confirmed by a pediatric allergist at enrollment; or (ii) when one of the parents had been diagnosed with asthma and/or allergic rhinitis by a physician, showing house dust mite-specific IgE over 1.0 kU/L by CAP-FEIA immunoassay (Pharmacia, Uppsala, Sweden).

Treatment of either probiotics or placebo was allocated by trials coordinator without detailed knowledge of the clinical history according to computerized randomization. The groups were stratified and block-randomized in accordance with (1) maternal allergy (allergy vs. no allergy), (2) older sibling's eczema, and (3) number of parents affected by allergic disease (1 vs. 2) (Table 1).

Mothers in the probiotics group took a mixture of *Bifidobacterium bifidum* BGN4 [1.6×10^9 colony forming units (CFU)], *Bifidobacterium lactis* AD011 (1.6×10^9 CFU), and *Lactobacillus acidophilus* AD031 (1.6×10^9 CFU) in 0.72 g of maltodextrin and 0.8 g of alpha-corn (Bifido Inc., Hongchungun, Korea) once daily from 8 wks before the expected delivery to 3 months after delivery. Infants were fed the same powder dissolved in breast milk, infant formula, or sterile water from 4 to 6 months of age. Mothers and infants in the placebo group took maltodextrin and alpha-corn without probiotic bacteria. The

Table 1. Baseline characteristics of the participants at the time of randomization

	Probiotics group n (%)	Placebo group n (%)
Enrolled number	57	55
Mother's age (yrs)*	29.93 ± 0.37	29.53 ± 0.45
Maternal allergic diseases	43 (75.4)	45 (81.8)
Parental history of allergic disease		
Biparents	22 (38.6)	14 (25.5)
Single parent	33 (57.9)	38 (69.1)
Allergic diseases in participant's family		
Eczema	34 (59.6)	38 (69.1)
AR or asthma	39 (68.4)	27 (49.1)
House dust mite-specific IgE of parents (kU/L)*		
D. pteronyssinus	6.63 ± 1.37	9.60 ± 2.56
D. farinae	10.41 ± 2.52	12.71 ± 3.01
Paternal smoking	14 (24.6)	19 (34.5)

*Mean ± s.e.m.

There were no significant differences between the groups for any of the variables determined by Student's *t* test for continuous data and Pearson's chi-square test for all nominal data.

AR, allergic rhinitis.

probiotic and placebo sachets and contents looked, smelled, and tasted identical. Compliance was monitored by recording the date at which the administration period was discontinued, and counting the remaining sachets. All mothers were requested to breastfeed their infants for at least 3 months after birth; Thereafter, they were permitted to feed their infants with cow's milk formula. Lactating mothers and infants were prevented from eating peanuts and eggs, as well as yogurt and other probiotic functional foods, during the course of the study.

Subjects were excluded if they met any one of the following exclusion criteria: (1) premature babies delivered at less than 36 wks of gestation; (2) infants with immune deficiency disease, necrotizing enterocolitis, or congenital disorders; or (3) those requiring anticancer treatment or a central venous catheter. In the current study, one infant with a congenital disorder was excluded.

The study protocol was approved by the Ethical Committee at the Samsung Medical Center. Written informed consent was obtained from all participants. Our study protocol was registered in ISRCTN (International Standard Randomised Controlled Trial Number). The registration number is ISRCTN26134979.

Clinical assessments

Infants were followed up to 1 yr of age, involving examinations at 3, 6, and 12 months to assess the occurrence of eczema, the main atopic disease during infancy. Diagnosis of eczema was confirmed when the skin lesions met the criteria of Hanifin and Rajka (16). The severity of eczema was determined using the Six Area Six Sign in Atopic dermatitis (SASSAD) score (17). We also collected data on various clinical histories such as gestational age, birth weight, cesarean section delivery, mother's age, duration of breastfeeding, diet, fever (≥38.5°C), respiratory tract infection, hospitalization, acute gastroenteritis, and use of antibiotics. Diagnosis and clinical assessment of eczema was performed by a pediatric allergist who remained unaware of the actual treatment administered during the entire study period.

Venous blood was obtained at 12 months of age to measure total IgE and specific IgE against common food allergens (egg white, cow's milk, wheat, peanut, soybean, and buckwheat) using CAP-FEIA (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Antigen-specific IgE levels greater than 0.35 kU/L were considered positive. 'Probable egg allergy' was defined when the egg white-specific IgE level was 2 kU/L or higher based on the 95% positive predictive value (18). Likewise, infants were diagnosed as having 'probable cow's milk allergy' when they showed cow's milk-specific IgE of 5 kU/L or higher.

Statistical analysis

We anticipated that this high-risk population would have a 60% cumulative incidence of eczema at an early age, and that intervention with probiotics could reduce the proportion of cases developing allergic diseases to 30%. The study design had more than 80% power at the 5% significance level; the estimated size of each group was 55, which allowed for a 15% dropout rate.

Pearson's chi-square test was used to compare the prevalence of outcome variables and background factors between the two groups. Logistic regression analysis was performed to compare the prevalence of eczema and food allergy, and multivariable logistic regression was used to adjust for potential confounding factors (cesarean delivery, breastfeeding, use of antibiotics). The results are given as Odds Ratio (OR) with a 95% Confidence Interval (CI). Total IgE and allergen-specific IgE of parents were normally distributed and assessed using Student's *t*-test, expressed as mean and standard error of the mean (s.e.m.). All statistical analyses were performed using SPSS 12.0K for Windows (SPSS Inc., Chicago, IL, USA). A p value of < 0.05 was considered to be statistically significant.

Results

Characteristics of the participants

A flow chart to show the progress of the present study is displayed in Fig. 1. Among the 159 pregnant women who took a blood test and filled in the questionnaire, 112 who met the inclusion criteria were randomized and divided into the probiotics (n = 57) and placebo (n = 55) groups. Prenatally, no mother discontinued administration of the sachets; overall, 90% of the sachets were administered in both groups. During the postnatal period, two infants in the probiotics group and one in the placebo group were excluded because of poor compliance (less than 70%); approximately 85% of the sachets were administered, with no difference in compliance between the groups. The actually treated prenatal periods were 53.62 ± 1.75 days in probiotics group and 52.29 ± 1.33 days in placebo group. The actual postnatal period of treatment was 180.94 ± 5.21 days in probiotics group and

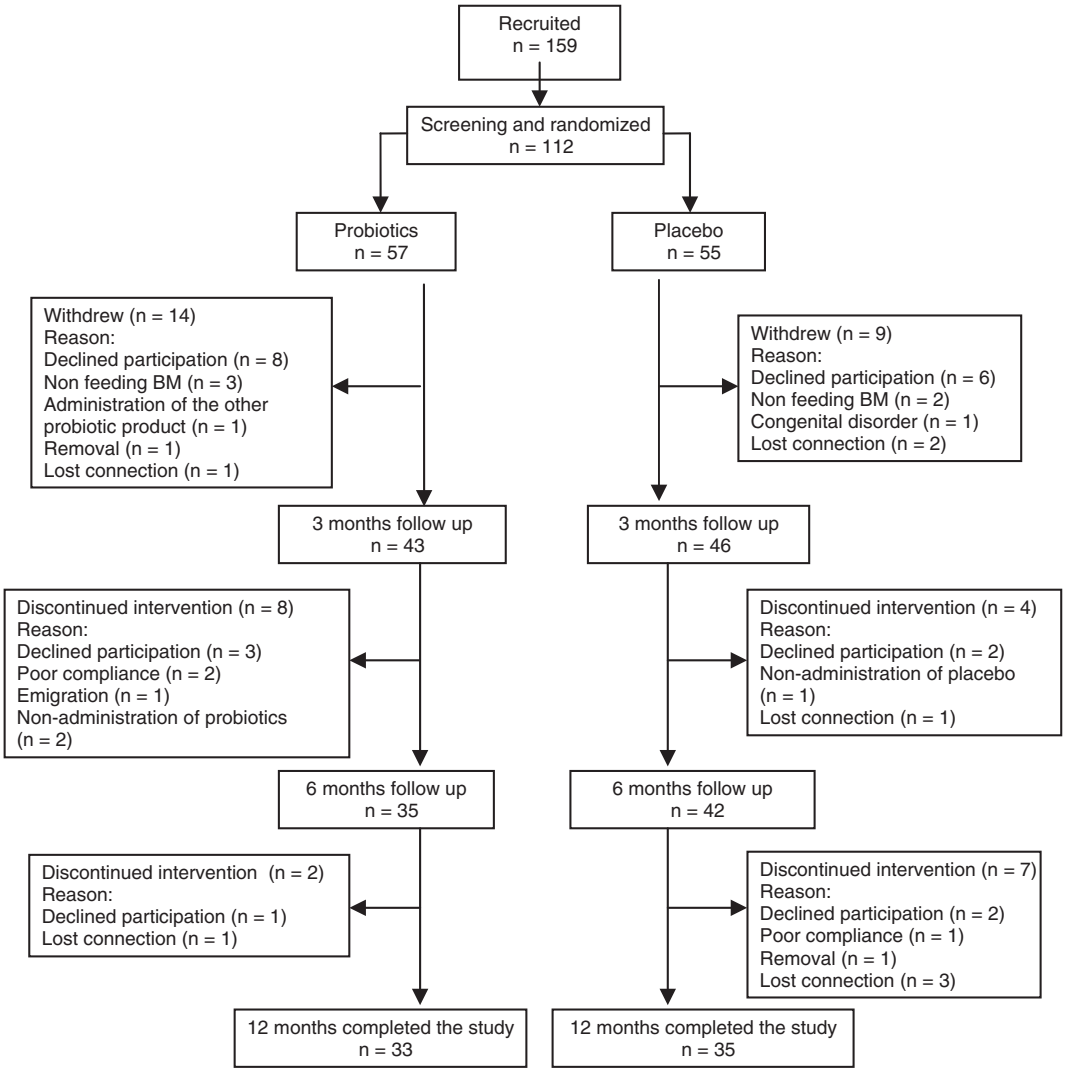

Fig. 1. Flow chart showing the progress of the study.

181.03 ± 5.06 days in placebo group. A total of 44 participants (39.3%) discontinued and ultimately, 68 mothers and their babies (33 in the probiotics group, 35 in the placebo group) completed the study (i.e., participated until the baby was 12 months of age).

There was no difference at the time of randomization between the two groups with respect to mother's age, maternal allergic diseases, biparental allergic diseases, family history of eczema, paternal smoking, or house dust mite-specific IgE levels of parents (Table 1). There was also no significant difference in terms of gestational age, birth weight, gender, presence of older siblings, infections, antibiotic use, and hospitalization during infancy between the two groups when only those participants successfully followed to 12 months were included (Table 2). The rate of cesarean delivery in the probiotics group was half that in the placebo group (15.2% vs. 31.4%; $p = 0.114$), and the total duration of the breastfeeding in the probiotics group was longer than in the placebo group (9.41 ± 0.61 vs. 7.69 ± 0.70 months; $p = 0.068$). Although these findings were not statistically significant, they were considered as potential confounding factors in subsequent analyses.

In the current study, the parents were asked to report any adverse effects whenever they happen. No serious adverse effects developed and although non-specific mild symptoms developed, these were unlikely to have been related to the administration of probiotics.

Effects of probiotics on development of eczema

The prevalence of eczema was not significantly different between the two groups at 3 months of

Table 2. Base and clinical characterization of infants who completed the 1 yr-study

	Probiotics group n (%)	Placebo group n (%)
Number at the end of study	33	35
Gestational age (wks)*	39.66 ± 0.25	39.47 ± 0.19
Birth weight (kg)*	3.33 ± 0.07	3.25 ± 0.06
Gender (boys/girls)	15/18	15/20
Cesarean section delivery	5 (15.2)	11 (31.4)
Duration of breast-feeding (month)*		
Exclusive breast-feeding	5.97 ± 0.89	5.26 ± 0.88
Total breast-feeding	9.41 ± 0.61	7.69 ± 0.70
Presence of older sibling	4 (12.1)	6 (17.1)
Infections during follow-up		
Fever (≥38.5°C)	13 (39.4)	11 (31.4)
Respiratory tract infection	26 (78.8)	26 (74.3)
Acute gastroenteritis	11 (33.3)	7 (20.0)
Antibiotics use during follow-up	13 (39.4)	11 (31.4)
Hospitalization during follow-up	6 (18.2)	6 (17.1)

*Mean ± s.e.m.
There were no significant differences between the groups for any of the variables determined by Student's *t* test for continuous data and Pearson's chi-square test for all nominal data.

age, up to which point lactating mothers were given the probiotics or placebo (probiotics group, 18.6% vs. placebo group, 34.8%; p = 0.086). At 6 months of age, when the infants were receiving formula supplemented with probiotics or placebo, the prevalence rate of eczema in the probiotics group was half that in the placebo group (20.0% vs. 40.5%; p = 0.053). At 12 months of age, the prevalence rate of eczema in the probiotics group was reduced to less than half that in the placebo group; this result was statistically significant (18.2% vs. 40.0%; p = 0.048; Table 3).The cumulative incidence of eczema in the probiotics group was significantly lower than that in the placebo group at 12 months of age (36.4% vs. 62.9%; p = 0.029; Table 3). This result demonstrated that probiotics protected the infants from developing eczema.

The severity of skin lesions was assessed only in those with eczema by SASSAD, and compared between the two groups. Those without eczema were not scored. There was no significant difference in the severity of eczema between the two groups.

IgE sensitization

Sera were obtained from 31 infants in the probiotics group and 29 in the placebo group to measure total and specific IgE; eight mothers (two from the probiotics group, six from the placebo group) refused to withdraw blood from their infants. Total IgE level and the frequency of sensitization against common food allergens was similar in both groups, suggesting that probiotics did not affect sensitization (Table 4). The prevalence rate of IgE-associated eczema or atopic eczema in the probiotics group was half that in the placebo group, although this was not significantly different (9.7% vs. 20.7%, p = 0.233). 'Probable egg allergy' or 'probable cow's milk allergy' occurred similarly in both groups (Table 4).

Discussion

In this double-blind, randomized, placebo-controlled study, we demonstrated the preventive effect of mixed probiotics (*B. bifidum* BGN4, *B. lactis* AD011, and *L. acidophilus* AD031) on development of eczema in infants at high risk of atopic diseases. *B. bifidum, B. lactis*, and *L. acidophilus* have been detected in samples from healthy humans (19, 20), and these probiotic bacteria were used in our study. *B. bifidum* BGN4 exhibited a prominent adhesive capacity for intestinal epithelial cells, which is one of the desirable properties for a probiotic effect (21). In the CD4 + $CD45RB^{high}$ T cell transfer inflammatory bowel disease model, *B. bifidum*

Table 3. Cross-sectional prevalence and cumulative incidence of eczema at 3, 6, and 12 months of age

	Probiotics group	Placebo group	p-value	Adjusted OR(95% CI)	p-value
Cross-sectional prevalence					
3 months	8/43 (18.6%)	16/46 (34.8%)	0.086	0.511 (0.178–1.468)	0.212
6 months	7/35 (20.0%)	17/42 (40.5%)	0.053	0.377(0.119–1.197)	0.098
12 months	6/33 (18.2%)	14/35 (40.0%)	0.048*	0.256(0.067–0.976)	0.046†
Cumulative incidence at 12 months	12/33 (36.4%)	22/35 (62.9%)	0.029*	0.243(0.075–0.792)	0.019†

*Significant difference between the groups as determined by Pearson's chi-square test.
†p value was calculated by multivariable logistic regression analysis adjusted for the antibiotics use, total duration of breastfeeding, and delivery by cesarean section.

Table 4. Comparison of sensitization and prevalence of probable food allergy between two groups

	Probiotics group n (%)	Placebo group n (%)	p-value
Number who completed blood test	31	29	
Total Serum IgE (kU/L)*	111.7 ± 58.9	80.6 ± 28.7	0.638
Food-specific IgE (≥0.35 kU/L)			
Any food	12 (38.7)	15 (51.7)	0.311
Egg white	9 (29.0)	8 (27.6)	0.901
Cow's milk	9 (29.0)	11 (37.9)	0.465
Soybean	3 (9.7)	3 (10.3)	0.931
Wheat	2 (6.5)	2 (6.9)	0.946
Peanut	1 (3.2)	4 (13.8)	0.139
Buckwheat	1 (3.2)	2 (6.9)	0.514
IgE-associated eczema	3 (9.7)	6 (20.7)	0.233
Probable food allergy			
Egg	4 (12.9)	4 (13.8)	0.919
Cow's milk	1 (3.2)	0 (0.0)	0.329

*Mean ± s.e.m.
There were no significant differences between the groups for any of the variables determined by Student's *t* test for continuous data and Pearson's chi-square test for all nominal data.

BGN4-fed mice showed suppression of inflammatory cell infiltration and reduced levels of CD4 + T lymphocyte infiltration and inflammatory cytokine production compared with skim milk-fed mice (22). Orally administered *B. bifidum* BGN4 also prevented IgE-mediated ovalbumin-induced allergy in C3H/HeJ mice (23). These findings suggest that *B. bifidum* BGN4 supplementation could be helpful in the control of aberrant immune responses.

In our preliminary study, mice fed with *B. lactis* AD011 and *L. acidophilus* AD031 showed suppressed levels of ovalbumin-specific IgE in serum, reduced concentrations of IL-4 and increased concentrations of IL-10 and IFN-γ in spleen cell culture assay on ovalbumin-induced allergy model (24). Additionally, *B. lactis* AD011 and *L. acidophilus* AD031 increased the production of IL-10 in dendritic cells (not published). Previous studies also have reported that the administration of *B. lactis* or *L. acidophilus* species alleviated the symptoms of allergic diseases. For example, supplementation with *B. lactis* Bb-12 reduced the severity of atopic eczema in infants (25), and oral administration of fermented milk containing *L. acidophilus* L-92 improved allergic rhinitis (26). Based on these results, we attempted to prevent the development of eczema with mixture of beneficial probiotics (*B. bifidum* BGN4, *B. lactis* AD011, and *L. acidophilus* AD031), and found these probiotics could be used for the infants at high risk of developing eczema.

Recent study revealed that supplementation with *L. acidophilus* (LAVRI-A1) did not prevent atopic dermatitis, but instead led to an increased frequency of common antigen sensitization in infants at high risk of allergy by 1 yr of age (15). This contradictory result may be attributed to different study population, the use of different strain or no prenatal administration of probiotics, despite using the same species, *L. acidophilus*. However, further studies are necessary to verify which species or strains are most beneficial, because our study used mixture of those probiotics, not a single strain.

Several clinical trials have investigated whether prenatal or indirect supplementation with probiotics via breastfeeding could enhance the primary prevention of eczema at an early age. When prenatal and breastfeeding mothers were supplemented with *L. rhamnosus* GG from 2–4 wks before delivery, the rate of their infants' atopic eczema at 2 yrs of age was half that of those supplemented with the placebo (13). Infants born from mothers supplemented with *L. reuteri* for 4 wks before delivery showed a lower frequency of IgE-associated eczema and positive reaction to a skin prick test than the placebo group (27). The above results may well suggest that maternal immunocompetence has an effect *in utero* or on breastfeeding infants. Recent study showed that maternal probiotic supplementation reduced sensitization in infants at high risk of developing allergic diseases and that might have been related to the change in the composition of breast milk such as TGF-β2 (28).

In this prospective study, the frequency of positive food antigen-specific IgE sensitization and food allergy in Korean infants at high risk was not reduced by supplementation with probiotics. This result was consistent with that of the previous study (29), although the reason for the discrepancy between development of eczema and sensitization against food allergens was not clear. However, our data showed that sensitization against any one of common food allergen appeared to be lower in probiotics group (38.7%) than in placebo group (51.7%). The prevalence of IgE-associated eczema also seemed lower in probiotics group (9.7% vs. 20.7%). Still it might be possible to demonstrate the effect of probiotics on the prevention of sensitization if investigations with larger study population are conducted.

Our study is limited by the high drop-out rate. Some participants stopped participating in the study without explanation, or moved from the area. In addition, we excluded all participants who did not adhere to our protocol. Consequently, a high proportion of participants failed

to complete the study. Intention-to-treat analysis may be helpful in such a situation; however, the full application of intention-to-treat analysis is possible only when complete outcome data are available for all randomized subjects. In the present study, however, data are missing regarding the primary outcome for discontinued participants, and the methods employed to deal with this problem were inadequate, potentially leading to bias. We analyzed a total of 44 participants who discontinued or withdrew from the study. Twenty-three participants dropped out before the first follow-up, and their development of eczema was not identified. Among 21 infants who discontinued the study after 3 months of age, the occurrence of eczema was 20.0% (2/10) in the probiotics group and 54.5% (6/11) in the placebo group at their last visit. Considering that the prevalence or cumulative incidence of eczema in the probiotics group is less than half that in the placebo group, the administration of probiotics in the discontinued participants appears to have had a similar preventive effect to that in those who completed the study. In addition, among those who successfully completed the 1-yr study, there was no difference in baseline characteristics or drop-out rate between the probiotics group and placebo group. Therefore, our results suggest that a probiotic mixture was beneficial in preventing eczema, despite the high drop-out rate.

This study showed high prevalence of eczema. The prevalence (40.0%) at 1 yr and cumulative incidence (62.9%) of eczema in placebo group seemed high in the present study. Other studies also showed that the frequency of eczema in placebo group was as high as 45–46% (10, 13). We obtained similar data in our previous study where the prevalence of eczema was 59% and 41% in cow's milk formula-fed group and breastmilk-fed group, respectively, in infants at high risk of eczema (30). Probably high incidence of eczema might have been due to the selection of infants with high risk of developing atopic disease.

Additionally, our study ended when the infants were 1 yr of age. Although we could find lower incidence of eczema in probiotics group, it is not clear whether this preventive effect persists as they grow older.

In conclusion, mixture of probiotics (*B. bifidum* BGN4, *B. lactis* AD011, and *L. acidophilus* AD031) have beneficial effect to prevent development of eczema in infants at high risk during their first year of life. Further studies are needed to understand the basic mechanisms of probiotics in the primary prevention of eczema.

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Short Note

The Efficacy of *Bifidobacterium longum* BORI and *Lactobacillus acidophilus* AD031 Probiotic Treatment in Infants with Rotavirus Infection

Myeong Soo Park [1], Bin Kwon [2], Seockmo Ku [3] and Geun Eog Ji [2,4,*]

1 Department of Hotel Culinary Arts, Yeonsung University, Anyang 430-749, Korea; mspark@yeonsung.ac.kr
2 Research Center, BIFIDO Co. Ltd., Hongcheon 250-804, Korea; 531083@hanmail.net
3 Fermentation Science Program, School of Agribusiness and Agriscience, College of Basic and Applied Sciences, Middle Tennessee State University, Murfreesboro, TN 37132, USA; Seockmo.Ku@mtsu.edu
4 Department of Food and Nutrition, Research Institute of Human Ecology, Seoul National University, Seoul 151-742, Korea
* Correspondence: geji@snu.ac.kr; Tel.: +82-2-880-6282

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Abstract: A total of 57 infants hospitalized with rotavirus disease were included in this study. The children were randomly divided into the study's two treatment groups: three days of the oral administration of (i) a probiotics formula containing both *Bifidobacterium longum* BORI and *Lactobacillus acidophilus* AD031 (N = 28); or (ii) a placebo (probiotic-free skim milk, N = 29) and the standard therapy for diarrhea. There were no differences in age, sex, or blood characteristics between the two groups. When the 57 cases completed the protocol, the duration of the patients' diarrhea was significantly shorter in the probiotics group (4.38 $\pm$ 1.29, N = 28) than the placebo group (5.61 $\pm$ 1.23, N = 29), with a p-value of 0.001. Symptoms such as duration of fever (p = 0.119), frequency of diarrhea (p = 0.119), and frequency of vomiting (p = 0.331) tended to be ameliorated by the probiotic treatment; however, differences were not statistically significant between the two groups. There were no serious, adverse events and no differences in the frequency of adverse events in both groups.

Keywords: probiotics; rotavirus; *Bifidobacterium*; *Lactobacillus*

1. Introduction

Diarrhea-associated deaths in children under five years old in developing countries have been a major cause of childhood mortality [1]. These illnesses are caused by multiple factors, including infections by pathogenic microorganisms, viruses, and parasites [2]. Among the many acute diarrheal diseases, infections caused by rotavirus may be more fatal in infants than in adults [3]. Global reports show that most babies and toddlers are infected with rotavirus by the age of five [4]. This causes serious problems in developing and/or low-income countries (e.g., South Asian and sub-Saharan African countries), and hundreds of thousands of babies are killed by rotavirus annually [5]. Recently, the developments of rotavirus vaccines (e.g., RotaTeg and Rotarix) have dramatically reduced the number of outbreaks in many countries and were proven safe; however, concerns remain regarding the cost of the rotavirus vaccines and their limited effectiveness in some cases [6]. Accordingly, supported therapeutic methods that are compatible with common rotavirus medical treatments and effectively relieve its symptoms should be developed.

A number of studies have identified the effect of several probiotic species (e.g., *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Propionibacterium*, *Saccharomyces* and *Streptococcus*) in the treatment and prevention of intestinal infections [7]. These probiotic bacteria have been shown to inhibit intestinal disease [8–11]. *Bifidobacterium* and *Lactobacillus* spp. are the most common bacteria and are considered the most beneficial probiotic organisms [12].

Although multiple probiotic microorganisms could be utilized in rotavirus treatments, some studies have not identified any significant therapeutic effects; therefore, the underlying mechanisms of the therapeutic effects of probiotics in humans are still unclear [13]. Studies have shown that some probiotic bacteria have little or no statistically significant effect on rotavirus [14,15]. Moreover, we can deduce that the effect of probiotics may vary based on the type of microorganism administered to the host. We aim to determine the efficacy of a commercially available probiotic product containing two probiotic cell types, i.e., *Bifidobacterium longum* BORI and *Lactobacillus acidophilus* AD031, in infants and/or toddlers with rotavirus-associated symthoms.

2. Materials and Methods

Design: All participants' guardians completed written, informed consent forms prior to the clinical experiment. All patients were recruited and classified from the inpatient Department of Pediatrics at Yonsei University Hospital in Seoul, Korea. This double-blind, randomized, and placebo-controlled clinical study tests the efficacy of probiotics formula to ameliorate the pathological symptoms in children hospitalized with rotavirus infections. The criteria applied to the experimental subjects are as follows: nine- to 16-month-old male and female infants were diagnosed as infected with rotavirus via a latex agglutination test. A total of 57 infants hospitalized with rotavirus infection were enrolled in this study. 28 patients were assigned to the probiotics treatment group, and the remaining 29 patients were assigned to the placebo group. The probiotics group was fed probiotic formula containing *B. longum* BORI and *L. acidophilus* AD031.

Diet and probiotic microorganisms: The probiotic powder contained two lyophilized probiotic species. Each probiotic packet contained 20 billion CFU/g of *B. longum* BORI and two billion CFU/g of *L. acidophilus* AD031 in powder form. The probiotics-free skim milk powder (placebo packet) was not visually distinguishable from the composite probiotic packet. Both the probiotic and placebo packets were supplied by BIFIDO Co., Ltd. (Hongchun, Korea). Each participant consumed the packets (i) twice a day (ii) for a total of three days (iii) within 10 min of each meal.

Statistical analysis: Paired *t*-tests were performed to assess the quantitative changes in the symptoms of rotavirus infection: duration of fever, frequency of diarrhea, frequency of vomiting, and duration of diarrhea before and after the study period in both groups. Results were considered statistically significant when the *p*-values were < 0.05.

3. Results and Discussion

A total of 57 infants hospitalized with rotavirus infection were enrolled in this study. Twenty-eight patients were randomly assigned to the probiotics group and 29 to the placebo group. The probiotics group was fed a probiotic formula containing *B. longum* BORI and *L. acidophilus* AD031. There were no differences in the age, sex, or blood characteristics of the two groups. The experimental outcomes are summarized in Table 1. The probiotics group showed a slightly reduced duration of fever ($p = 0.119$), frequency of diarrhea ($p = 0.119$), and frequency of vomiting ($p = 0.331$) compared to the placebo group; however, these differences were not significant. By contrast, the duration of diarrhea during the three-day treatment showed a significant difference between the probiotics group (4.38 ± 1.29) and the placebo group (5.61 ± 1.23) with a *p*-value of 0.001 (Table 1). There were no serious, adverse events and no difference in the frequency of adverse events in both groups.

Table 1. Duration and frequency of rotavirus-associated symptoms in patients treated with probiotics and placebo.

Symptoms		Condition		*p*-Value
Category	**Parameter**	**Placebo (*N* = 29)**	**Probiotics (*N* = 28)**	
Duration	Fever	4.32 ± 1.94	3.66 ± 1.14	0.119
(Days)	*Diarrhea*	*5.61 ± 1.23*	*4.38 ± 1.29*	*0.001*
Frequency	Vomiting	1.82 ± 0.94	1.55 ± 1.12	0.119
(Times/Day)	Diarrhea	2.64 ± 0.73	2.38 ± 0.49	0.331

The probiotic formula containing *B. longum* BORI and *L. acidophilus* AD031 utilized in this work is likely be an effective adjuvant to relieve acute diarrhea caused by rotavirus. Several studies showed that various strains of probiotic bacteria, such as *L. reuteri* and *L. rhamnosus*, were effective in managing acute diarrhea caused by rotavirus in toddlers. In the present experiment, the efficacy of *B. longum* BORI and *L. acidophilus* AD031 probiotic products was tested. Our rationale for the *L. acidophilus* and *B. longum* combination was based on the general microbial composition, which shows a predominance of *Lactobacillus* sp. in the small intestine and *Bifidobacterium* sp. in the large intestine (among a variety of beneficial bacteria present in healthy human subjects). Eighteen of 23 clinical trials of probiotic formulas resulted in mitigating acute diarrhea, and the reduction of the duration of diarrhea in the studies' probiotics treatment group was reported to be 0.5 to 1.5 days [16]. The duration of diarrhea may vary depending on a child's health status, diet, and prescribed medication. Our study demonstrated a statistically significant diarrhea reduction of 1.2 days. The efficacy of probiotics is strain-specific, so this may be due to the use of different strains in different studies. Basu et al. [17] conducted a clinical study with 10^7 CFU/day LGG and concluded that it was not effective, but when they performed the same study again [18] with 10^{10} and 10^{12} CFU/day LGG, they concluded that a higher concentration of LGG administration in acute diarrhea patients was effective in reducing the diarrhea frequency, diarrhea period, and hospitalization period. Fang et al. [19] reported a dose-dependent effect of *Lb. rhamnosus* on fecal rotavirus concentration and suggested 6×10^8 CFU/day as the minimal effective dose, which was similar to the data of Guanidalin [20], who concluded that at least 10 billion cells/day was necessary. Dubay [21] also applied the commercially available probiotic formula (VSL#3, CD Pharma India, New Delhi, India) to mitigate acute diarrhea, which showed a more rapid recovery compared to the control group and decreased the necessity of electrolyte treatments. In contrast to the positive results mentioned above, a probiotic formula containing 10^9 CFU/day of *B. lactis* and 10^8 CFU/day of *S. thermophilus* failed to decrease the duration of rotavirus diarrhea [22]. These contrasting results suggest that further clinical experiments are necessary in order to understand the scientific basis of the efficacy of probiotics and its relation to a number of criteria the strain of probiotics, the type of rotavirus, the severity of the symptoms, the ages and races of the children, etc. Further study using animal models also should be considered since the experimental conditions in this model can be better controlled [23–27].

4. Conclusions

The results of the present study demonstrated that a probiotic formula containing *Bifidobacterium longum* BORI and *Lactobacillus acidophilus* AD031 reduced the duration of rotavirus diarrhea in young Korean children.

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Author Contributions: Myeong Soo Park and Bin Kwon designed the experiment under the supervision of Geun Eog Ji. Myeong Soo Park and Bin Kwon performed the research. Myeong Soo Park, Seockmo Ku and Geun Eog Ji analyzed the data and wrote the manuscript. All authors were involved in the revision and editing of this work.

Conflicts of Interest: The authors declare no conflict of interest.

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ORIGINAL ARTICLE

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Effect of Probiotics on Symptoms in Korean Adults with Irritable Bowel Syndrome

Kyoung Sup Hong*, Hyoun Woo Kang*, Jong Pil Im*, Geun Eog Ji[†,‡], Sang Gyun Kim*, Hyun Chae Jung*, In Sung Song*, and Joo Sung Kim*

*Department of Internal Medicine, Seoul National University College of Medicine, [†]Department of Food and Nutrition, Research Institute of Human Ecology, Seoul National University College of Human Ecology, Seoul, [‡]Research Institute, BIFIDO Co., Ltd., Hongchun, Korea

Background/Aims: Irritable bowel syndrome (IBS) is a troublesome disease. Some strains of probiotics reportedly exert remarkable immunomodulatory effects, and so we designed a prospective double-blind randomized placebo-controlled clinical study to assess their effects in Korean adults with IBS. **Methods:** IBS patients who met Rome III criteria were randomly assigned to receive composite probiotics or placebo. A total of 20 billion lyophilized bacteria were administered twice daily for 8 weeks. Primary outcome variables were symptom scores consisting of abdominal pain, flatulence, defecation discomfort, and sum of symptom scores. A visual analogue scale was used to quantify the severity. Secondary outcome variables consisted of the quality of life and bowel habits including defecation frequency and stool form. **Results:** Thirty-six and 34 patients were randomized to the probiotics and placebo groups, respectively. Intention-to-treat analysis showed significant reductions in pain after 8 weeks of treatment: −31.9 and −17.7 in the probiotics and placebo groups, respectively (p=0.045). The reductions in abdominal pain, defecation discomfort, and sum of scores were more significant in 58 patients with a score of at least 3 on the baseline stool-form scale. **Conclusions:** Composite probiotics containing *Bifidobacterium bifidum* BGN4, *Lactobacillus acidophilus* AD031, and other species are safe and effective, especially in patients who excrete normal or loose stools. **(Gut and Liver 2009;3:101-107)**

Key Words: Probiotics; Irritable bowel syndrome; *Bifidobacterium bifidum*

INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most troublesome diseases, which has the high prevalence as well as the chronic and recurrent course. In United States, IBS is known as the most common gastrointestinal disease and comprises 25 to 50% of all referrals to gastroenterologists,[1] and the prevalence of IBS has estimated to range 9% to 22% of the population.[2] In Korea, Park *et al.*[3] reported the prevalence of IBS increased up to 16.8%. A few effective medicines such as cisapride, tegaserod and alosetron have been withdrawn from the market because of their serious adverse drug reactions. There is no specific treatment that has proven to be effective and safe in the patients with IBS.

Through previous studies, IBS is known to be associated with low-grade inflammation of the intestinal mucosa regardless of whether to be the post-infectious subtype or not.[4,5] Although a few clinical studies to evaluate the immunomodulatory effect of probiotics showed symptom relief in IBS patients, probiotics are not yet used widely in daily practice.[6-8] As global competetion for searching more potent strain was heating up, a well-designed clinical study becomed necessary to validate the effect of promising probiotics. Two *in vitro* studies using some strains of *Bifidobacterium bifidum* demonstrated that they were effective in inhibiting lipopolysaccharide (LPS)-induced inflammation, and the later study showed high LPS-binding capacity and inhibition of inflammatory cytokine.[9,10] Kim *et al.*[11] reported *Bifidobacterium bifidum* BGN4 strain had the significant immunomodulatory effect

Correspondence to: Joo Sung Kim
Department of Internal Medicine, Seoul National University College of Medicine, Seoul National University Hospital, 28, Yeongeon-dong, Jongno-gu, Seoul 110-744, Korea
Tel: +82-2-740-8112, Fax: +82-2-743-6701, E-mail: jooskim@snu.ac.kr
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on the control of inflammatory bowel disease (IBD) using a mouse model. With composite probiotics including *Bifidobacterium bifidum* BGN4 and other promising strains, we designed a prospective double-blind randomized placebo-controlled clinical study to prove the effect in the Korean adults with IBS.

MATERIALS AND METHODS

1. Study population

Patients were recruited from the outpatient department of Seoul National University Hospital. The study protocol was approved by the institutional review board. The inclusion criteria were as follows: age of 19-75 years, both male and female, and the presence of previous gastrointestinal symptoms suggestive of IBS using the Rome III criteria regardless of its subtypes. All participants gave a written informed consent form that had been approved by the institutional review board.

The exclusion criteria were as follows: previous abdominal surgery except appendectomy and hernia repair, history of IBD, current use of medications that may alter gastrointestinal motility, antibiotics or probiotics within 2 weeks prior to the 1-week run-in period, severe co-morbidity such as cancer, heart or renal failure, gynecologic disease etc., and pregnant or breast-feeding female.

2. Study design

We performed a parallel-group, double-blinded, randomized, placebo-controlled clinical study. A 1-week run-in observation period was followed by an 8-week treatment period. During this entire 9-week period, participants were required to record a daily diary of bowel habits consisting of frequency and consistency. A questionnaire on irritable bowel symptoms such as abdominal pain, flatulence and defecation discomfort was recorded at baseline, 4th and 8th week after treatment. A questionnaire on quality-of-life (QOL) was recorded at baseline and 8th week.

Primary outcome variables were symptom scores that consisted of abdominal pain, flatulence, defecation discomfort and the sum of these three symptom scores. Laborious evacuation, tenesmus and urgency were included in questionnaire for defecation discomfort. A 100 mm visual analogue scale (VAS) was used to measure the severity of each symptom as scores ranging from 0 to 100. When participants were asked to mark VAS after treatment, they could look at his or her previous marks. Secondary outcome variables were bowel habits that recorded using a validated Bristol stool form scale and QOL that recorded using a RAND 36-item health survey.[12-14]

3. Administration of probiotics

Composite probiotics were composed of 4 viable lyophilized bacteria species: *Bifidobacterium bifidum* BGN4; *Bifidobacterium lactis* AD011; *Lactobacillus acidophilus* AD031; and *Lactobacillus casei* IBS041. Each probiotic packet with equal doses of 4 strains contained total 20 billion lyophilized bacteria in a powder form. A placebo packet containing skim milk powder looked identical to the composite probiotics. Both probiotics and placebo were supplied by BIFIDO Co., Ltd., Hongchun, Korea. Each participant in both treatment groups received one packet orally with water within 10 minutes after a meal, twice daily (40 billion lyophilized bacteria per day) for 8 weeks.

4. Randomization

We used blocked randomization method with block size 4 or 6 and generated permutations at random using SPSS for Windows 12.0.1 (SPSS Inc., Chicago, IL, USA) provided by the medical research collaborating center at our institution that was independent of medical care. All participants were assigned an allocation number in regular sequence after confirmation of enrollment. For adherence to double-blind design, the allocation number was matched to a randomization code successively by a clinical trial pharmacist. Till completion of the study, the clinical trial pharmacist kept the randomization table sealed off.

5. Sample size calculation and statistical analyses

On the ground of previous reports, we assumed the response rates would be 70% in probiotics group and 40% in placebo group.[8] The response was defined as reduction of symptom score by at least 50% after treatment. Other assumptions for sample size calculation were as follows: alpha error 0.05; statistical power 0.8; drop-out rate 0.05; and one-sided test. We used the equation below, the sample size was estimated as 35 per group in view of drop-out rate.

$$N = \frac{\left(Z_\alpha \sqrt{2p(1-p)} + Z_\beta \sqrt{p_1(1-p_1) + p_2(1-p_2)}\right)^2}{d^2}$$

p_1 (response rate in probiotics group)=0.7
p_2 (response rate in placebo group)=0.4
$p=(p_1+p_2)/2=0.55$
$d=p_2-p_1=0.3$
$Z_\alpha=1.65$ (alpha error=0.05)
$Z_\beta=0.84$ (statistical power=0.8)

All data were collected by a single trained interviewer who was a clinical research coordinator. Week 0 (the end

of run-in phase) was considered as baseline in all statistical analyses. The "intent-to-treat" (ITT) population was defined as all participants who received probiotics or placebo for at least one week and visited our hospital for the interview once or more. Efficacy analysis was performed in the ITT population. χ^2-test was performed to test response rates. As symptom score, QOL score, consistency and frequency were all continuous variables, two-sided T-test was performed using the 0.05 significance level.

RESULTS

Between 1 November 2007 and 29 February 2008, 76 patients were screened. 5 patients (6.5%) were ineligible as they did not meet the inclusion criteria, and 1 withdrew consent. A total of 70 patients were enrolled, 36 were randomized to probiotics group and 34 to placebo. Demographic and clinical characteristics were similar between the two groups (Table 1).

1. Compliance and concomitant medications

Of 36 participants assigned to probiotics, 35 completed treatment as planned. One participant withdrew from the study due to an exacerbation of abdominal pain associated with IBS. Another one participant, who was assigned to placebo, withdrew from the study due to an exacerbation of constipation associated with IBS. Both withdrawn participants were included in efficacy analysis based on the definition of ITT population; symptom scores and the bowel habit were imputed using the mean value of the group. A total of 68 participants completed the study. Overall compliance was more than 98% in both groups.

Eleven of 70 participants required concomitant medications during the study, 3 in probiotics group and 8 in placebo. Ten participants, except one in probiotics group who was prescribed a common cold medication, used loperamide, prokinetics, pain killers, histamine 2 receptor antagonists, proton pump inhibitors or laxatives due to an exacerbation of bowel symptoms. According to ITT principle, all the eleven patients were included in analyses.

2. Symptom scores, QOL and bowel habits

ITT analyses showed significant reductions of pain score after 8 weeks of treatment (−31.9 in probiotics group vs. −17.7 in placebo [p=0.045]) and defecation discomfort after 4 weeks of treatment (−29.2 vs. −13.5, respectively [p=0.043]). Subgroup analyses in 58 patients whose baseline Bristol stool form scales were 3 or more

Table 1. Characteristics of Subjects

Characteristics	Probiotics (n=36)	Placebo (n=34)
Age (years)		
Mean (±SE)	36±2	38±3
Range	21-69	22-72
Females (n)	11	12
Baseline score (±SE)		
Pain	50.3 (±3.5)	46.9 (±3.4)
Flatulence	49.9 (±3.6)	49.3 (±4.8)
Defecation	53.5 (±4.0)	47.0 (±5.1)
Sum	153.6 (±8.9)	143.2 (±9.0)
QOL	104.6 (±1.1)	104.7 (±1.4)
Defecation frequency*	8.1	7.1
Stool consistency†	4.2	4.0
IBS subtype‡		
Diarrhea	19	13
Constipation	7	7
Mixed	2	4
Unsubtyped	8	10

QOL, quality-of-life.
*Bowel movements per week, †Bristol stool form scale; average for a week, ‡Rome III criteria.

Table 2. Symptom Scores Analyzed over 4 and 8-week Treatment Period (Two-sided T-test Using the 0.05 Significance Level)

		Probiotics	Placebo	p-value
ITT population (n=70)				
Pain	Baseline	50.3	46.9	0.487
	Δ 4 week	−23.9	−10.9	0.061
	Δ 8 week	−31.9	−17.7	0.045
Flatulence	Baseline	49.9	49.3	0.928
	Δ 4 week	−18.5	−18.4	0.982
	Δ 8 week	−27.0	−21.3	0.437
Defecation	Baseline	53.5	47.0	0.311
	Δ 4 week	−29.2	−13.5	0.043
	Δ 8 week	−30.5	−18.4	0.122
Sum	Baseline	153.6	143.2	0.413
	Δ 4 week	−71.7	−42.8	0.115
	Δ 8 week	−89.5	−57.5	0.064
Subgroup* (n=58)				
Pain	Baseline	51.2	46.2	0.315
	Δ 4 week	−26.9	−5.8	0.004
	Δ 8 week	−33.9	−13.3	0.006
Flatulence	Baseline	49.2	47.8	0.826
	Δ 4 week	−19.6	−13.0	0.463
	Δ 8 week	−26.7	−15.9	0.175
Defecation	Baseline	53.2	45.0	0.826
	Δ 4 week	−30.4	−10.6	0.013
	Δ 8 week	−30.0	−14.5	0.064
Sum	Baseline	153.6	138.9	0.288
	Δ 4 week	−76.9	−29.4	0.010
	Δ 8 week	−90.6	−43.6	0.010

*Baseline Bristol stool form scale ≥3.

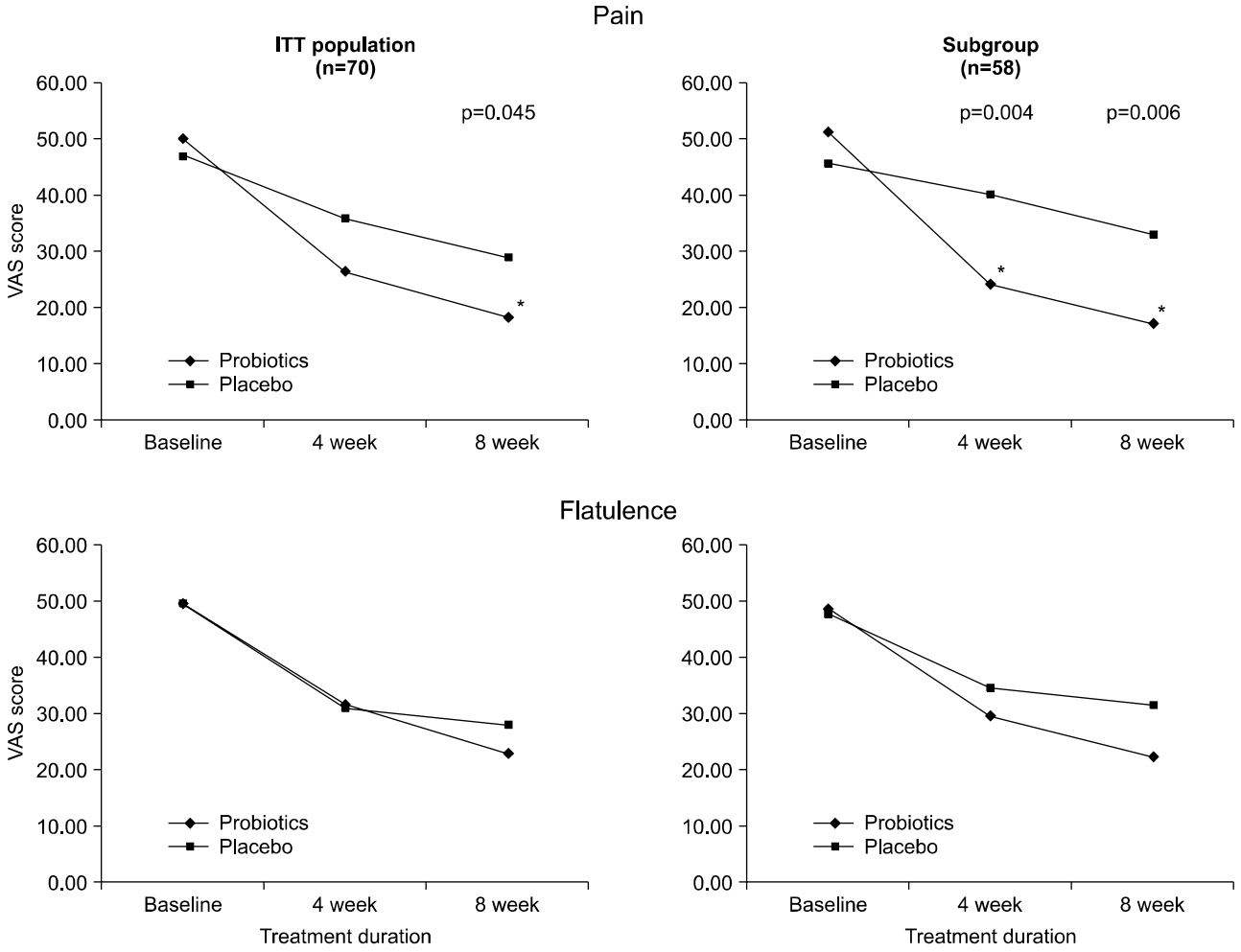

Fig. 1. Pain and flatulence scores analyzed over 4- and 8-week treatment periods.

showed more significant reductions of pain score after 8 weeks of treatment (−33.9 in probiotics group vs. −13.3 in placebo [p=0.006]), defecation discomfort score after 4 weeks of treatment (−30.4 vs. −10.6, respectively [p=0.013]), and sum of scores after 8 weeks of treatment (−90.6 vs. −43.6, respectively [p=0.010]) (Table 2, Figs. 1 and 2). Subgroup analyses in 10 patients, whose baseline Bristol stool form scales were below 3, did not show any significant changes. Response rate evaluation through χ^2-test failed to show significant changes as follows: response rate in pain were 64% in probiotics group vs. 44% in placebo (p=0.248), response rate in defecation discomfort were 58% vs. 41% (p=0.317), and response rate in sum of scores were 56% vs. 50% (p=0.750), respectively. There was no significant change of QOL and bowel habits including defecation frequency and stool consistency in both groups (Table 3).

3. Adverse events

There was no serious adverse event associated with treatments. Twelve of 70 participants reported mild adverse events including common cold, headache, cystitis, low back pain etc. The number of adverse events per group was 8, same in both groups.

DISCUSSION

Symptoms of IBS are subjective and there is no objective test that can measure severity of IBS. Symptom scores are popular methods for assessing severity of IBS, but these can be influenced by an interviewer as well as patients themselves. Strict double-blind design is essential to assess the effect of probiotics on symptoms in IBS patients. We could perform a strict double-blinded study by the help of MRCC and clinical trial pharmacists for random code generation and code-matching. Patel *et al.*[15]

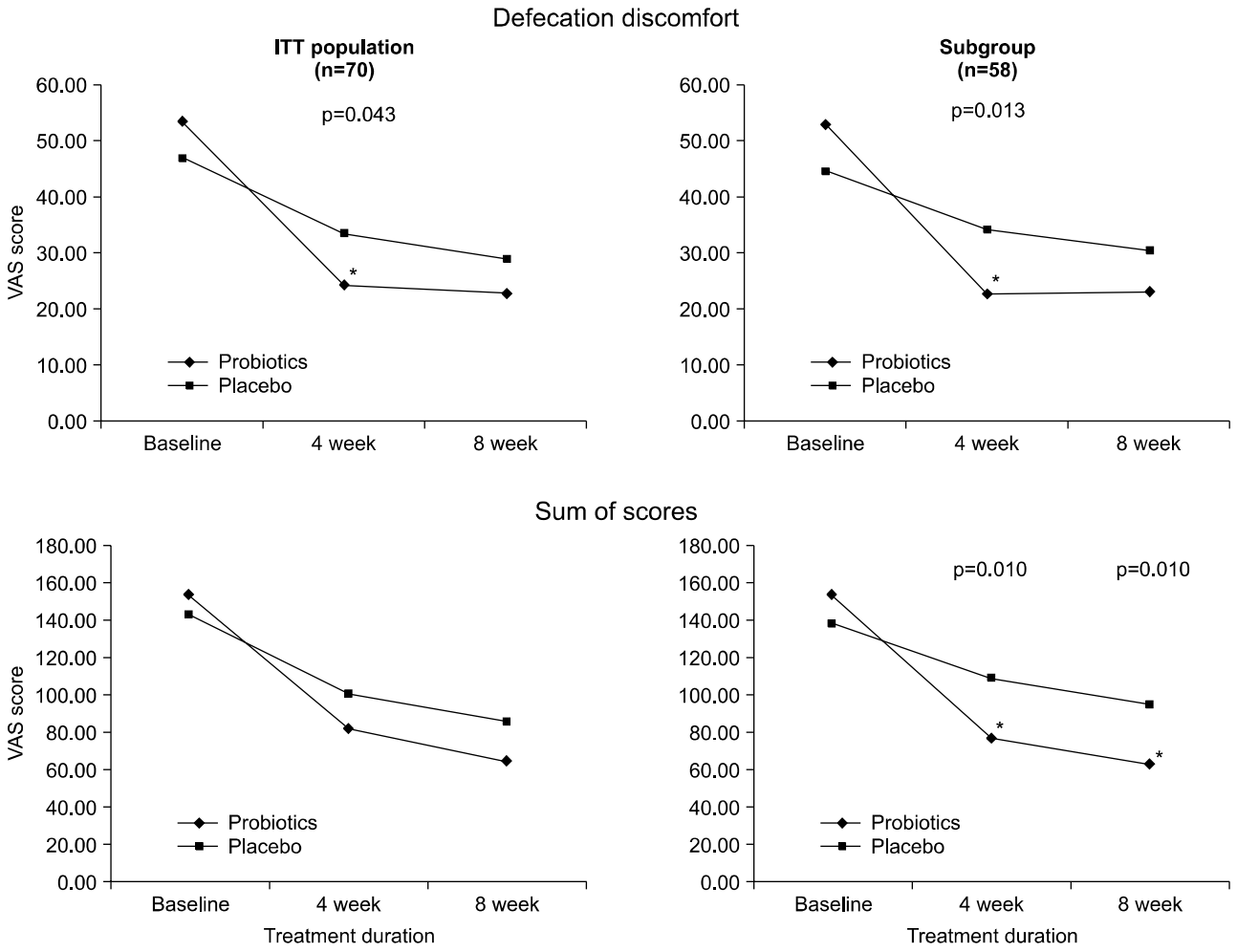

Fig. 2. Defecation discomfort and sum of scores analyzed over 4- and 8-week treatment periods.

Table 3. Analysis of Quality of Life, Defecation Frequency and Stool Consistency over 8-week Treatment Period (Two-sided T-test Using the 0.05 Significance Level)

		Probiotics	Placebo	p-value
ITT population (n=70)				
QOL	Baseline	104.6	104.7	0.957
	⊿ 8 week	−1.3	−1.1	0.887
Frequency†	Baseline	8.1	7.1	0.414
	⊿ 8 week	−0.1	0.1	0.817
Consistency‡	Baseline	4.2	4.0	0.453
	⊿ 8 week	0.2	−0.1	0.430
Subgroup* (n=58)				
QOL	Baseline	104.4	104.8	0.876
	⊿ 8 week	−1.5	−1.7	0.919
Frequency†	Baseline	8.7	7.9	0.553
	⊿ 8 week	−0.6	−0.2	0.689
Consistency‡	Baseline	4.6	4.4	0.374
	⊿ 8 week	−0.1	−0.4	0.241

QOL, quality-of-life.
*Baseline Bristol stool form scale ≥3, †Bowel movements per week, ‡Bristol stool form scale; average for a week.

reported that placebo response in IBS studies ranged from 16% to 71% via meta-analysis. In our study, the overall placebo effect was 35% that is comparable with many other IBS studies and seems to be a matter of course in double-blinded study.[16-18] Strong points of our study design include strict double-blind design, data collection by a single trained interviewer, relatively large number of study population (n=70) and no need of data processing including adjusting and standardization.

Although Kim *et al.*[6,7] reported that VSL #3 reduced flatulence scores in the patients with IBS, probiotics have not been the standard treatment of IBS due to the following reasons: need of huge dose of probiotics (VSL #3, 4.5×10^{11} bacteria/packet), relatively low efficacy (10 mm difference from placebo on 100 mm scale) and no effect in abdominal pain and urgency. A recent report showed that low dose of one strain (*Bifidobacterium infantis* 35624, 1×10^{8} bacteria/capsule) reduced symptom scores in IBS patients,[8] and the result suggested that the effect of probiotics for IBS was dependent on a specific strain as well

as dose. Some strains of *Lactobacillus* were known to be effective in controlling IBS symptoms.[19] A paper studying an alteration of gut microbiota reported that *Lactobacillus* sequences were absent in stool from IBS patients, contrary to healthy control.[20] Regarding the reduction of abdominal pain by probiotics, a recent study demonstrated that one strain of Lactobacillus acidophilus induced the expression of mu-opioid and cannabinoid receptors in intestinal epithelial cells of rodents and mediated analgesic functions in the gut.[21] As described in introduction, considering the immunomodulatory effect of *Bifidobacterium bifidum* BGN4 and potential benefit of *Lactobacillus* sp. we selected study medication as composite probiotics composed of *Bifidobacterium bifidum* BGN4, *Bifidobacterium lactis* AD011, *Lactobacillus acidophilus* AD031 and *Lactobacillus casei* IBS041. All of them are original strains that were collected from Koreans and have been never used in clinical study.

We demonstrated that selected composite probiotics were effective in IBS patients (ITT population, n=70) as follows: pain reduced by 64% in probiotics group vs. 38% in placebo (p=0.045), and defecation discomfort reduced by 55% vs. 29% (p=0.043), respectively. In agreement to a previous report, probiotics were more effective in patient who excreted mainly normal or loose stool (baseline Bristol stool form scale $\geq$3, n=58) as follows: pain reduced by 66% in probiotics group vs. 29% in placebo (p=0.006), defecation discomfort reduced by 57% vs. 24% (p=0.013), and sum of scores reduced by 59% vs. 31% (p=0.010), respectively.[8] In contrast to many reports that probiotics showed minimal or no effect on abdominal pain, we demonstrated the beneficial effect in the treatment of IBS symptoms including abdominal pain using composite probiotics containing *Bifidobacterium bifidum* BGN4 and *Lactobacillus acidophilus* AD031.[6-8,16] Although Sinn *et al.*[22] reported that two strains of *Lactobacillus acidophilus* reduced abdominal pain by 20% more than placebo in IBS patients, we could demonstrate superior effect of probiotics on abdominal pain up to 37% over placebo. As compared with VSL #3 that showed effectiveness on overall score up to 16% over placebo, our composite probiotics were more effective on overall score up to 28% over placebo.[6]

χ^2-test was performed to test the response which was defined as reduction of symptom score by at least 50% after treatment in this study, but it could not show significant change. Data loss was inevitable in the process of converting symptom scores into responder status which was classified as yes or no, and it seemed to be the reason of low sensitivity of χ^2-test. By simultaneously doing parametric analyses over changes of individual scores after treatment, we could demonstrate the effect of probiotics accurately.

Although the analyses on bowel habits showed slight decrease of frequency, there was no statistically significant change between two groups, which might be due to low power of this study. A previous study showed normalization of frequency in *Bifidobacterium*-treated group through *post hoc* analyses (n=182) using data stratified by baseline bowel movements per day.[8] On the basis of further large-scale studies, probiotics are expected to be revealed as effective in correction of bowel habits.

In conclusion, composite probiotics containing *Bifidobacterium bifidum* BGN4, *Bifidobacterium lactis* AD011, *Lactobacillus acidophilus* AD031 and *Lactobacillus casei* IBS041 were safe and effective, especially in patients who excreted mainly normal or loose stool.

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Probiotic treatment induced change of inflammation related metabolites in IBS-D patients/double-blind, randomized, placebo-controlled trial

Jinjoo Kim[1,2] · Kumsun Cho[3] · Joo Sung Kim[1] · Hyun Chae Jung[1] · Bumsik Kim[4] · Myeong Soo Park[5] · Geun Eog Ji[5] · Joo-Youn Cho[3,6] · Kyoung Sup Hong[7]

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Abstract There have been many studies suggesting that probiotics are effective in patients with diarrhea-predominant irritable bowel syndrome (IBS-D). However, its mechanism of action as well as prediction of response is still to be elucidated. In the present study, to find out metabolomic characteristics of probiotic effect in IBS-D, we compared IBS symptom changes and metabolomic characteristics in the subjects' urine samples between multi-strain probiotics (one strain of *Lactobacillus* sp. and four strains of *Bifidobacterium* sp.) group (n = 32) and placebo group (n = 31). After 8 weeks' administration (3 times/day), dissatisfaction in bowel habits and stool frequencies were significantly improved. Also, probiotics group had significantly changed seven metabolites including palmitic acid methyl ester (PAME) and 4,6-dihydroxyquinoline, 4-(2-aminophenyl)-2,4-dioxobutanoic acid (DOBA). According to IBS-SSS and IBS-QoL questionnaires, IBS-SSS responders showed higher PAME levels and IBS-QoL responders showed lower DOBA levels. This suggests potential role of these metabolites as a biomarker to predict probiotics effect in IBS-D patients.

Keywords Irritable bowel syndrome · Probiotics · Metabolomics

Introduction

Irritable bowel syndrome (IBS) is common (Gwee et al., 2009), but the precise pathophysiology is still need to be elucidated (Barbara et al., 2009; Camilleri, 2013; Ghoshal and Ranjan, 2011; Hammerle and Surawicz, 2008; Jeffery et al., 2012; Keszthelyi et al., 2012; Malagelada and

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✉ Kyoung Sup Hong
kshong1@empas.com

Jinjoo Kim
jjkim0727@gmail.com

Kumsun Cho
kscho615@snu.ac.kr

Bumsik Kim
bumik@yeonsung.ac.kr

Myeong Soo Park
bifidopark@bifido.com

Joo-Youn Cho
joocho@snu.ac.kr

[1] Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine and Hospital, Seoul 03080, Republic of Korea

[2] Department of Internal Medicine, Gyeongsang National University School of Medicine, Jinju, Gyeongnam 52727, Republic of Korea

[3] Department of Clinical Pharmacology and Therapeutics, Seoul National University College of Medicine and Hospital, Seoul 03080, Republic of Korea

[4] Department of Food and Nutrition, Yeonsung University, Anyang, Gyeonggi 14011, Republic of Korea

[5] Bifodo Co., Hongcheon, Gangwon 25117, Republic of Korea

[6] Cancer Research Institute, Seoul National University College of Medicine, Seoul 03080, Republic of Korea

[7] Department of Gastroenterology, Mediplex Sejong Hospital, 20, Gyeyangmunhwa-ro, Gyeyang-gu, Incheon 21080, Republic of Korea

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Malagelada, 2016; Spiller, 2004). Pharmacologic, psychologic, and complementary approaches are considered as therapeutic options in IBS patients (Quigley, 2012) and probiotics are one of those options.

Probiotics alter the composition of the gut flora by exerting antibacterial, antiviral and anti-inflammatory effects at mucosal surface. One of the most active research areas is about the mechanisms how the efficacy of probiotics shows in some IBS patients (Harper et al., 2018; O'Hara and Shanahan, 2007). One of possible action is anti-inflammatory effects of probiotics (Dunne et al., 1999). For example, in an experimental animal with interleukin (IL)-10 knockout model of colitis, both a *Lactobacillus* and a *Bifidobacterium* showed a marked and parallel reduction in inflammation in the colon and the pro-inflammatory cytokines, such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-12, while levels of the anti-inflammatory cytokine, such as transforming growth factor (TGF)-β, were maintained (McCarthy et al., 2003). We also have shown that *B. bifidum* BGN4, *B. lactis* AD011 and *Lb. acidophilus* AD031 were effective to improve symptoms in IBD patients who excrete normal or loose stools (Hong et al., 2009).

Recently, metabolomic approaches have studied systematic metabolism related to several diseases, such as inflammatory bowel diseases (IBD) and celiac disease (Bertini et al., 2009; Marchesi et al., 2007; William et al., 2009; Yu et al., 2018). Some metabolic changes in serum, urine and feces are known to be associated with the gut microflora, intestinal absorption, and host energy metabolism. However, there are only few studies with IBS patients, so far.

The aim of present stud was to examine the effect of a multi-species probiotics mixture on IBS symptoms and metabolomics characteristics. First, we compared IBS symptom changes between probiotics and placebo groups. Secondly, we tested effects of a multi-species probiotics mixture on metabolomic characteristics in the subjects' urine samples and if there are any metabolomics differences between probiotics and placebo group responders and non-responders.

Materials and methods

Patients

Patients were enrolled prospectively at the Department of Gastroenterology of Seoul National University Hospital, between March 2013 and May 2013. The inclusion criteria were: (a) age between 18 and 75 years, (b) diagnosed with diarrhea-dominant IBS according to Rome II criteria (c) without any organic abnormalities by physical and laboratory examination during the screening period. Exclusion criteria were: (a) intolerance to probiotics or lactose, (b) pregnancy or lactation, (c) severe systemic illness (liver cirrhosis, congestive heart failure, chronic renal failure, angina, uncontrolled hypertension, endocrine disorder, metabolic disorder, or malignant tumors), (d) history of inflammatory bowel disease or psychiatric disorder, (e) alcohol or drug addiction, (f) previous abdominal surgery other than appendectomy, (g) being judged ineligible for participation in clinical trials by clinicians.

Signed informed consent was obtained from each patient prior to the enrolment. The study was conducted according to the Declaration of Helsinki and was approved by the Institutional Review Boards of Seoul National University Hospital (ClinicalTrials.gov Identifier: NCT01637714).

Study design

Figure 1 is a flow chart of the study design. Patients fulfilling the inclusion criteria were checked with the baseline (week 0) stool form, stool frequency, and visual analogue scale (VAS). After urine samples were collected, patients were randomized to receive either placebo or a multi-species probiotic mixture through a computer-generated table. All patients and investigators, except for the study coordinator, were kept blinded to allocation until study completion.

Participants visited the clinic to get their investigational products and were examined for IBS symptoms at 4 weeks and 8 weeks after the first administration. At these visits, patients submitted self-administered questionnaires on stool form/frequency and VAS score for each IBS symptom using IBS-SSS (severity scoring system) and IBS-QoL (quality of life) questionnaires. According to IBS-SSS, responders were defined as subjects who showed more than 50 points decrease after the probiotics treatment. And according to IBS-QoL, responders were defined as subjects who showed more than 10 points decrease after the probiotics treatment. At 4 and 8 weeks, we also collected a follow-up urine samples.

Study medication

Combined probiotics, which included 5 strains of probiotics (*Bifidobacterium longum* BORI, *Bifidobacterium bifidum* BGN4, *Bifidobacterium lactis* AD011, *Bifidobacterium infantis* IBS007, and *Lactobacillus acidophilus* AD031) were used in this study. Three strains BGN4, AD011 and AD031 were reported to be safe and effective, especially in IBD patients who excrete normal or loose stools by Kim et al. (Hong et al., 2009), and BORI and IBS007 were screened through preliminary animal test (data not shown). A capsule was composed of a total of

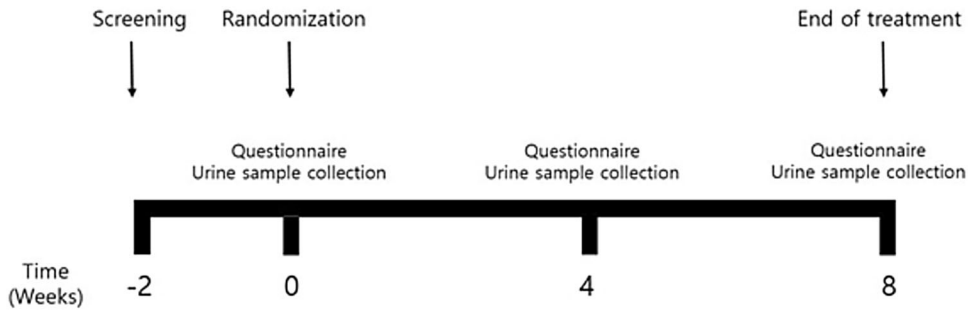

Fig. 1 A flow diagram illustrating the study design

5×10^9 viable cells in a lyophilized powder form with the other ingredients including maltodextrin, corn starch, and silicon dioxide. The placebo capsule had almost the same contents as the active medication, even though the bacteria were replaced with maltodextrin. Probiotics (n = 32) or placebo (n = 31) was administered 3 times daily.

Assessment of urine metabolites

Urine samples 100 μL with 100% cold H_2O 400 μL were vortexed for 10 min and centrifuged at 18,341×*g* for 20 min at 4 °C to remove particulates, 5 μL of which was injected into HPLC for the chromatographic separation. Chromatographic separations of metabolites in urine were performed with a Zorbax SB-C18, 50 × 2.1 mm, 1.8 μm (Agilent Technologies, Santa Clara, CA) analytical column using an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA): 2 mM ammonium formate in H_2O (0.1% formic acid) as a mobile phase A, 100% methanol (0.1% formic acid) as a mobile phase B, and flow rate was 0.4 ml/min. Q-TOF MS was done with Agilent Q-TOF 6530 mass spectrometer and the overall quality of the analysis procedure was monitored using repetition of a pooled urine sample (data not shown). The multivariate data matrix was analyzed by Mass Profiler Professional (MPP) software B.12.01 (Agilent Technologies) package which was used to principal component analysis (PCA). The identities of the specific metabolites were confirmed by using the human metabolome database (HMDB) and METLIN, and comparing chromatographic retention times and their mass spectra (MS/MS fragmentation patterns) to those obtained using commercially available reference standards. Levels of metabolites discriminating placebo and probiotics groups were normalized to creatinine levels in urine.

Statistical analysis

All statistical analyses were performed usingSPSS for Windows (ver. 18.0; SPSS, Chicago, IL). Student's t-tests was used for continuous variables and Chi square tests or Fisher's exact tests were used for categorical variables. To compare the quantitative changes of urine metabolites before and after the study period in both groups, paired t-tests were used. Spearman rank correlation coefficient were used to check the correlation. The results with a p value less than 0.05 were considered statistically significant.

Results and discussion

Baseline clinical characteristics and IBS symptom changes

A total of 63 patients were enrolled and randomized to probiotics (n = 32) or placebo (n = 31) group. There were no significant differences in the baseline clinical and laboratory characteristics between the probiotic a placebo groups (Supplementary Table 1). When comparing the effects of probiotics and placebo on IBS symptoms, probiotics significantly changed defecation discomfort (− 29.1 in the probiotics group vs. − 21.4 in the placebo group; $p = 0.0423$) and stool frequency (− 1.3 in the probiotics group vs. + 0.7 in the placebo group; $p = 0.0431$), however, in terms of other symptoms, there was no significant difference (Table 1). It might be due to the nature of IBS symptoms. It usually takes a long time to prove a symptomatic improvement to any treatment. According to 'FDA Guidance for Industry Irritable Bowel Syndrome—Clinical Evaluation of Drugs for Treatment'(US Department of Health and Human Services, 2012), a treatment period of at least 8 weeks duration was recommended at the section of trial design. In addition to the minimum duration of treatment, low numbers of subjects in the present study might be the cause of negative response in the IBS-SSS and IBS-QoL scores. The probiotics treatment resulted in better clinical relief in IBS patients by showing more IBS-SSS and IBS-QoL score decreases compared to those of placebo group; − 87.3 and − 14.5 in the probiotics group (42% and 21% improvements), and − 69.5 and − 11.5 in the placebo group (34% and 18% improvements), respectively (Fig. 2). However, the numbers of clinical responders, according to IBS-SSS

Table 1 Changes in symptom scores

	Placebo (n = 31)	Probiotics (n = 32)
Abdominal pain		
Baseline (± SD)	35.5 (± 8.9)	42.5 (± 11.4)
Δ 4 week	− 15.1	− 20.0
Δ 8 week	− 18.2	− 23.3
Bloating		
Baseline (± SD)	37.4 (± 15.1)	40.2 (± 18.9)
Δ 4 week	− 15.3	− 25.1
Δ 8 week	− 16.2	− 27.2
Defecation discomfort		
Baseline (± SD)	51.1 (± 22.8)	50.0 (± 20.5)
Δ 4 week	− 20.7	− 26.3
Δ 8 week	− 21.4	− 29.1*
Stool frequency		
Baseline (± SD)	8.1 (± 2.1)	7.5 (± 3.5)
Δ 8 week	0.7	− 1.3*
Stool consistency		
Baseline (± SD)	5.1 (± 2.2)	5.3 (± 1.2)
Δ 8 week	0.35	− 0.1

Abdominal pain, bloating, defecation discomfort, stool frequency, and stool consistency after probiotics and placebo administrations for 4 and 8 weeks

*$p < 0.05$ versus placebo group

or IBS-QoL scores, were not significantly different between those two groups (Table 2). Even though this association could not guarantee any definite causal relationships between them, this might be the valuable first step to elucidate the pathophysiology how probiotics alleviate IBS symptoms in some IBS-D patients. Many hypotheses have been advanced to explain the pathophysiology of IBS, including dysmotility, visceral hypersensitivity, aberrant cerebral representation of visceral events, and abnormal stress responses. However, none of them has provided definite explanations for all the IBS patients with various phenotypes (Quigley and Craig, 2012). More recently, the focus in IBS research has shifted to more local factors such as impaired gut barrier function, low-grade inflammation/immune activation, and an altered microbiome. Given its heterogeneous nature, it is likely that IBS involves more than one pathophysiological entity and may ultimately prove to encompass a number of distinct entities that cannot currently be separated on the basis of symptom clustering systems. Evidences have accumulated to suggest the presence of a low grade inflammatory state or immune activation in some IBS patients. Some findings in the colonic mucosa, such as increased mast cell numbers, enhanced mast cell degranulation, more intense lymphocyte infiltration, and an up-regulation of both pro-inflammatory cytokines and Toll-like receptors, supports this theory (Barbara and Cremon, 2008; Barbara et al., 2004; Brint et al., 2011; Chadwick et al., 2002; Crowell et al., 2004; Dinan et al., 2006; O'Mahony et al., 2005; O'Sullivan et al., 2000; Piche et al., 2008; Scully et al., 2010; Spiller et al., 2000; Talley and Butterfield, 1996; Weston et al., 1993).

Changes of urine metabolites after probiotics treatment

We performed untargeted metabolome analyses and generated principal component analysis (PCA) score plots to find metabolites characterizing different endogenous metabolome of probiotics administered IBS patients. Four and eight weeks' treatment of probiotics changed metabolome of IBS patients (Supplementary Fig. 1). There are 7 metabolites distinguishing metabolomic characteristics between probiotics and placebo groups: 2-ketobutyric acid, L-kynurenine, 4,6-dihydroxyquinoline, 4-(2-aminophenyl)-2,4-dioxobutanoic acid (DOBA), palmitic acid methyl ester (PAME), cholic acid, and palmitoleoyl ethanolamide (PEA) (Table 3). Among them, urinary levels of PAME and PEA were increased, and those of others were decreased with high significances ($p < 0.001$) except cholic acid after probiotics treatment for 8 weeks (Supplementary Fig. 2).

Comparison of urine metabolites between responders and non-responders

Patients treated with probiotics were divided into responders and non-responders by IBS-SSS or IBS-QoL scores differences from 0- to 8-week (ΔIBS-SSS and ΔIBS-QoL, respectively) (Table 2). According to responders criteria based on IBS-SSS and IBS-QoL (decrease more than 50 points and 10 points, respectively), we compared relative intensity differences of urine metabolites listed in Table 3. In the IBS-SSS responders group, urinary levels of PEA and PAME were increased and, DOBA, L-kynurenine and cholic acid were decreased after probiotics treatment for 8 weeks (Supplementary Fig. 3). In the IBS-QoL responders group, 4,6-dihydroxyquinoline, 2-ketobutyric acid and cholic acid were decreased after probiotics treatment for 8 weeks (Supplementary Fig. 4). Notably, L-kynurenine, 4,6-dihydroxyquinoline, and DOBA are associated with tryptophan metabolism. This metabolism is known for important in inflammatory bowel disease (Knights et al., 2013), and kynurenine pathway plays a key role in the tryptophan metabolism. Indeed, previous researches stated that 4,6-dihydroxyquinoline and DOBA were increased in IBD animal models (Zhang et al., 2012), and in our study, those metabolites were decreased after probiotics treatment

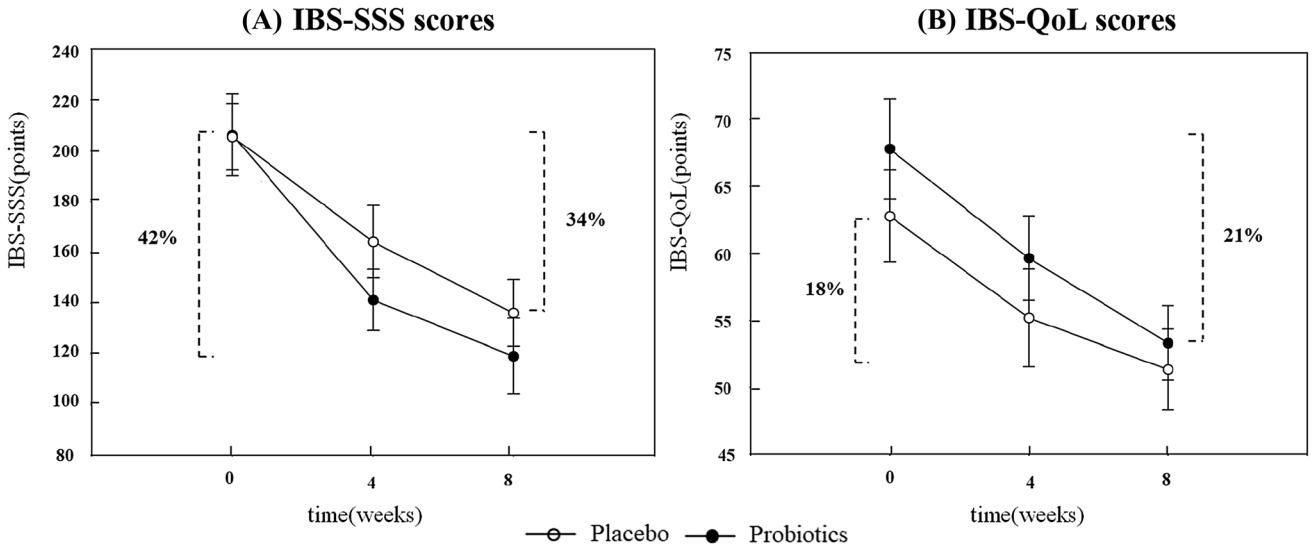

Fig. 2 Comparison of (**A**) IBS-SSS and (**B**) IBS-QoL scores between placebo and probiotics groups

Table 2 Comparison of responders and non-responders by IBS-SSS or IBS-QoL scores differences between 0- and 8-week in probiotics group

		Probiotics (n = 32)
ΔIBS-SSS		
Responders	≥ 50 points	21 (66%)
Non-responders	< 50 points	11 (34%)
ΔIBS-QoL		
Responders	≥ 10 points	19 (59%)
Non-responders	< 10 points	13 (41%)

in IBS patients. Saturated free fatty acids are known for inducing the expression of cyclooxygenase-2 (Lee et al., 2001), thus palmitic acid could increase inflammation as a member of them. However, this inflammatory effect could be ameliorated by adduction methyl ester to palmitic acid via microbial organisms (Cavigelli et al., 1995), and many studies elucidated that PAME decreases inflammation related interleukins, cytokines and prostaglandins (Cai et al., 2005), and PEA, a kind of N-acylethanolamines, also has been reported to have anti-inflammatory effects (Syed et al., 2012). Therefore, increased PAME and PEA might indicate anti-inflammatory effects of probiotics in IBS patients. Also, decreased cholic acid represents bile acid homeostasis stabilization since there are many studies demonstrated elevated cholic acid in IBD animal model and Crohn's disease patients (Tagesson et al., 1985; Zhang et al., 2012). And 2-ketobutyric acid, a well-known product of homocysteine degradation, was reduced after probiotics treatment. Given that homocysteine induces cell injuries as a results of inflammation (Schlüssel et al., 1995), reduced 2-ketobutyric acid might attributable to suppressed inflammation through homocysteine and 2-ketobutyric acid pathway.

Also, we performed correlation analysis to find metabolites associated with IBS improvements following probiotics treatment in patients. Among distinctive 7 metabolites between probiotics and placebo group, urinary levels of PAME showed significant correlations with IBS-SSS improvement in responders (Fig. 3A, R = 0.425;

Table 3 Endogenous metabolites discriminating the probiotics treatment group from the placebo group

Retention time (min)	m/z	Formula	Fold change (probiotics/placebo)	Identity
0.5	[101.0244]−	$C_4H_6O_3$	0.72	2-Ketobutyric acid
1.7	[207.0775]−	$C_{10}H_{12}N_2O_3$	0.36	L-Kynurenine
3.3	[162.0550]+	$C_9H_7NO_2$	0.59	4,6-Dihydroxyquinoline
3.6	[206.0459]−	$C_{10}H_9NO_4$	0.31	4-(2-Aminophenyl)-2,4-dioxobutanoic acid (DOBA)
10.2	[288.2982]+	$C_{17}H_{34}O_2$	1.77	Palmitic acid methyl ester (PAME)
11.2	[407.2803]−	$C_{24}H_{40}O_5$	0.48	Cholic acid
12.1	[320.2647]+	$C_{18}H_{35}NO_2$	2.26	Palmitoleoyl ethanolamide (PEA)

$p = 0.027$). And DOBA with IBS-QoL showed improvements in responders (Fig. 3C, R = − 0.534; $p = 0.011$).

However, in non-responders, there was no significnat correlation: R = 0.056; $p = 0.435$ for PAME, and R = − 0.056; $p = 0.431$ for DOBA (Fig. 3B, D). Compared with non-responders, IBS-SSS responders showed initially higher PAME levels and IBS-QoL responders showed initially lower DOBA levels (Fig. 3A, C).

As a conclusion, this study demonstrated that probiotics could improve IBS symptoms in IBS-D patients and this might be associated with alteration of metabolomes. Probiotics decreased more IBS-SSS and IBS-QoL than placebo (8%p and 3%p, respectively) and changed levels of urinary metabolites mainly relating to inflammation, therefore we could come up with an idea of probiotics' anti-inflammation effects (Fig. 2). However, not all patients were responsive to the probiotics treatment. This might be attributed to the heterogeneity of IBS patients and it would be clinically useful if we can predict who would be responsive to probiotics. In this aspect, this study suggest that urinary levels of metabolites can be the candidate biomarkers for this. Especially urinary PAME and DOBA levels showed significant correlation with clinical responsiveness. Moreover, urinary samples are much easier to get than stool samples. Several previous studies have already shown that not only stool samples but also urinary samples can reveal characteristics of intestinal environment quite precisely (Dabur et al., 2017; Marcobal et al., 2013).

However, this study had some limitations. First, even though probiotics group showed higher proportion of patients with adequate symptom relief than the placebo group, there were no significant differences between those

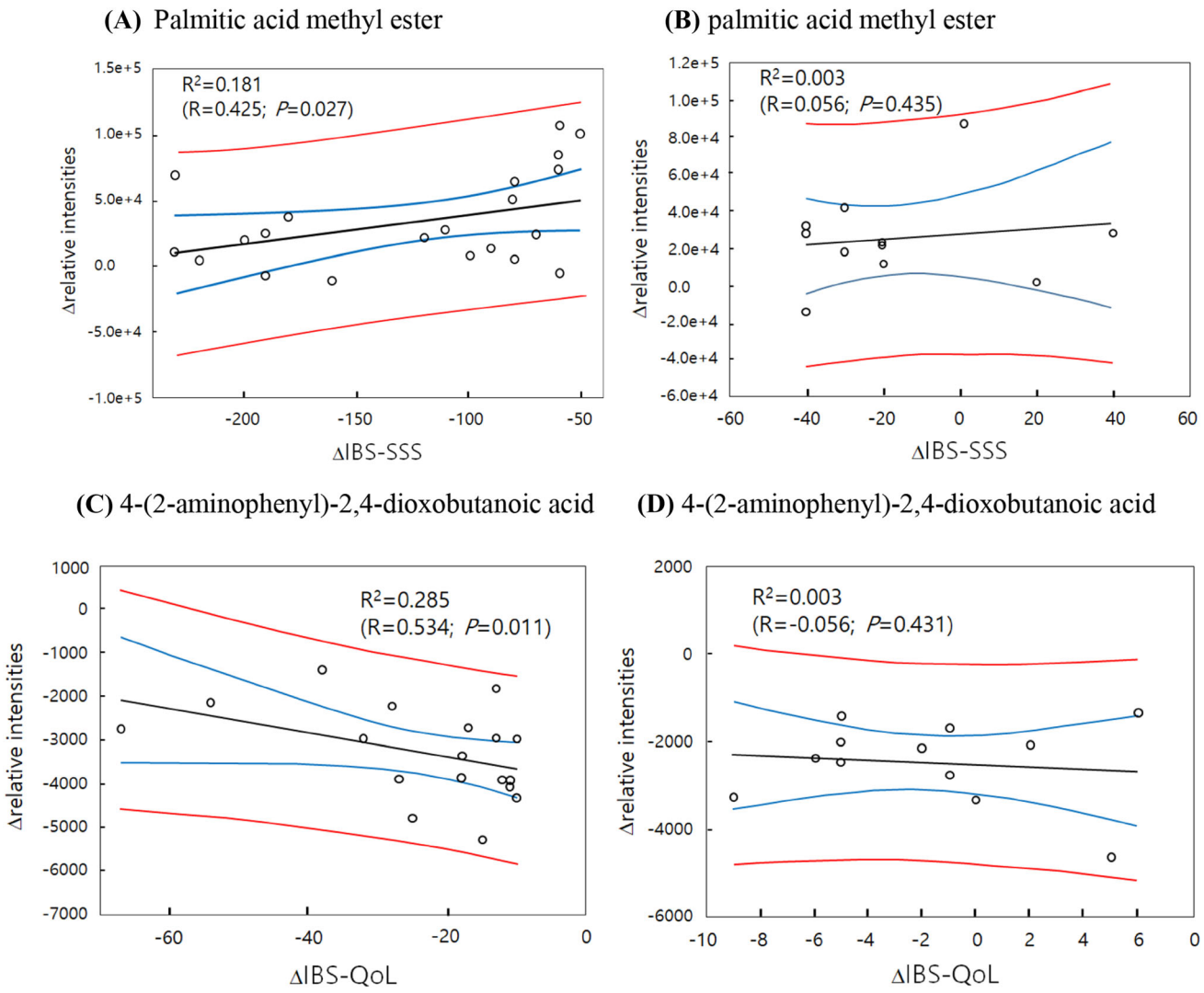

Fig. 3 Correlation analysis of metabolites and IBS-SSS or IBS-QoL in the probiotics treatment group. Palmitic acid methyl ester and IBS-SSS in responders (**A**) and in non-responders (**B**). 4-(2-Aminophenyl)-2,4-dioxobutanoic acid and IBS-QoL in responders (**C**) and in non-responders (**D**) excluding outliers. Black line: linear trend; blue line: 95% confidence band; red line: 95% prediction band

of IBS-QoL and IBS-SSS. This can be explained as a placebo effect because the placebo group in this study showed high rate of symptom relief. This remarkably high placebo effect would be one of major obstacles in assessing the efficacy of probiotics in IBS patients. Secondly, the diagnosis of IBS-D relies mostly on the clinical features and there is no objective diagnostic test or validated biomarker for IBS-D diagnosis. Thus, patients with heterogeneous disease entities could be included simply as IBS-D patients. Lastly, even though urine metabolites discriminating probiotics and placebo groups were found, we cannot be certain that it is the cause of IBS symptoms or the results of probiotics treatment. These changes could not guarantee any causal relationships. Therefore, more careful interpretation on these data would be mandatory and further well-designed studies with mechanism experiments in vitro or in vivo are needed to fully characterize metabolic characteristics and the effect of probiotic supplementation on metabolic modulation of IBS. Also, it is necessary to do microbiome related study in the future, because the probiotics treatment can cause major changes in the microbiome, and as a result, in the metabolome.

Nevertheless, the data presented in this study are valuable suggesting that in some IBS patients, probiotics can spur the anti-inflammatory process and this might lead to the symptom relief and these metabolites might be useful to predict who would respond to the probiotics. Furthermore, this study highlights the potential of untargeted metabolic profiling as a possible monitoring method for nutritional or pharmacological interventions.

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Compliance with ethical standards

Conflict of interest The authors have no financial conflicts of interest to declare.

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Original Article

Probiotic Supplementation Improves Cognitive Function and Mood with Changes in Gut Microbiota in Community-Dwelling Older Adults: A Randomized, Double-Blind, Placebo-Controlled, Multicenter Trial

Chong-Su Kim, PhD,[1] Lina Cha, MS,[1] Minju Sim, MS,[1] Sungwoong Jung, MD,[2] Woo Young Chun, PhD,[3] Hyun Wook Baik, MD, PhD,[4] and Dong-Mi Shin, PhD[1,5,*]

[1]Department of Food and Nutrition, College of Human Ecology, Seoul National University, Republic of Korea. [2]Seoul W Internal Medicine Clinic, Republic of Korea. [3]Department of Psychology, Chungnam National University, Daejeon, Republic of Korea. [4]Department of Internal Medicine, Clinical Nutrition and Metabolism, Bundang Jesaeng Hospital, Seongnam, Republic of Korea. [5]Research Institution of Human Ecology, Seoul National University, Republic of Korea.

*Address correspondence to: Dong-Mi Shin, PhD, Seoul National University, Seoul 08826, Republic of Korea. E-mail: shindm@snu.ac.kr

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Abstract

Probiotics have been proposed to ameliorate cognitive impairment and depressive disorder via the gut–brain axis in patients and experimental animal models. However, the beneficial role of probiotics in brain functions of healthy older adults remains unclear. Therefore, a randomized, double-blind, and placebo-controlled multicenter trial was conducted to determine the effects of probiotics on cognition and mood in community-dwelling older adults. Sixty-three healthy elders (≥65 years) consumed either placebo or probiotics containing *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI for 12 weeks. The gut microbiota was analyzed using 16S rRNA sequencing and bioinformatics. Brain functions were measured using the Consortium to Establish a Registry for Alzheimer's disease, Satisfaction with life scale, stress questionnaire, Geriatric depression scale, and Positive affect and negative affect schedule. Blood brain-derived neurotrophic factor (BDNF) was determined using enzyme-linked immunosorbent assay. Relative abundance of inflammation-causing gut bacteria was significantly reduced at Week 12 in the probiotics group ($p < .05$). The probiotics group showed greater improvement in mental flexibility test and stress score than the placebo group ($p < .05$). Contrary to placebo, probiotics significantly increased serum BDNF level ($p < .05$). Notably, the gut microbes significantly shifted by probiotics (*Eubacterium* and Clostridiales) showed significant negative correlation with serum BDNF level only in the probiotics group ($R_S = -0.37$, $R_S = -0.39$, $p < .05$). In conclusion, probiotics promote mental flexibility and alleviate stress in healthy older adults, along with causing changes in gut microbiota. These results provide evidence supporting health-promoting properties of probiotics as a part of healthy diet in the older adults.

Keywords: Probiotics, RCT, Gut microbiota, Cognitive function, Mood status, Healthy older adults

Aging is characterized by progressive decline in biological functions of the organism (1). The functions of the central nervous system also change during normal aging, leading to age-associated cognitive decline and mood disorders that are common and major health issues among older adults (1). Most industrialized countries are facing a rapid increase in the proportion of older adults considered to be in the danger zone of neurological diseases (1,2). Beyond the increasing risk of health issues, the critical social problems such as high economic burden and low growth potential of an aging society have ensued (2). Therefore, development of efficient preventative and therapeutic strategies targeting neurodegenerative disorders should be considered as a public health priority to promote healthy aging in the global population.

The gut microbiota, a collection of microorganisms found in the gastrointestinal tract, has pivotal roles in anatomical, physiological, and immunological host functions (3,4). The gut microbiota

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undergoes a significant transition in its composition and function during aging and these alterations can affect health and age-related diseases (5,6). Based on a series of studies, it is now becoming evident that maintaining gut microbial balance during aging is imperative for healthy late life (7). Recently, the emerging concept of gut–brain axis, referring to a bidirectional relationship between gut and brain, has linked gut microbiota to age-related neurodegenerative diseases, such as Alzheimer's disease, and mood disorders including depression and anxiety (8–12). The interplay between gut and brain involves a complex network of endocrinological, immunological, and neural mediators, which has been considered as a critical target for the manipulation of brain health and neurodegenerative diseases (13–15).

Diet is one of the critical lifestyle factors for physical and mental well-being throughout the life span, including later life (16,17). A growing body of evidence suggests that dietary components or nutrients affect various biological functions including brain activity (10,16,18–20). Therefore, research is actively focusing on the emerging concept of brain health preservation through dietary interventions. Probiotics, as part of a healthy diet, have received increasing attention for their potential to regulate brain health via the microbiota–gut–brain axis (9,21). Probiotic bacteria have been shown to affect intestinal microbial dynamics and homeostasis, and influence the physiology of the intestine and distal organs, including the brain (22). However, most of the current evidence comes from animal experiments, and it is crucial yet challenging to assess whether such findings can be translated to humans. Thus, it is critical to validate the clinical properties and effects of probiotics on human gut and brain health, particularly focusing on independently living older individuals, which can be majorly affected by cognitive and mental disorders. Therefore, we conducted a randomized, double-blind, placebo-controlled, multicenter trial to test our hypothesis that probiotic consumption has beneficial effects on intestinal health, and contributes to ameliorate cognitive and mental impairment in the older adults.

Materials and Methods

Study Design

The study was a randomized, double-blind, placebo-controlled, multicenter clinical trial examining the effects of probiotics consumption on intestinal and brain health in elders over the age of 65, conducted at Seoul National University (Seoul, Republic of Korea) and Bundang Jesaeng Hospital (Seongnam, Republic of Korea) from March 2018 to March 2019. The study included a 2-week wash-out period and a 12-week intervention period. During a 2-week wash-out phase, eligible participants were instructed to refrain from dietary supplements including probiotics and other dietary supplements. Participants were then randomly assigned to one of the two following groups: Placebo or Probiotics group. During the intervention period, participants consumed their assigned products twice a day for 12 consecutive weeks. They visited the clinic at baseline (Week 0), Week 4, Week 8, and Week 12 for a compliance check and blood and fecal samples were collected at each visit; and they conducted neuropsychological test at baseline, Week 4 and Week 12. Participants were asked not to change their usual dietary habits and health-related behaviors during the period of intervention. They were asked to record treatment intake, and unusual events such as the use of medication and experiencing adverse events in a daily manner in order to check the adherence to the study. This work is registered with CRiS (Clinical Research Information Service; http://cris.nih.go.kr; https://cris.nih.go.kr/cris/search/search_result_st01_en.jsp?seq=14020&ltype=&rtype=. Registration ID: KCT0003929).

Participants

Participants were recruited from communities in Seoul and Seongnam in the Republic of Korea. Recruitment flyer was posted at Gwanak-gu Community Health Center (Seoul, Republic of Korea), Seoul W Internal Medicine Clinic (Seoul, Republic of Korea) and Bundang Jesaeng Hospital (Seongnam, Republic of Korea). Candidates were invited to an onsite screening, which includes interviews asking about health history, health-related behavior, and dietary habits. Assessment of physical and cognitive functional status was conducted using activities of daily living, instrumental activities of daily living, and Mini-Mental State Examination (MMSE) by experienced research staff.

Criteria for eligibility

Eligible subjects had to be over 65 years old and to consent to be randomly assigned and refrain from consuming any other dietary supplements, which include other probiotics, yogurts with live, active cultures or supplements, and immune-enhancing supplements, during the period of the study. We excluded participants with the use of antibiotics, anti-inflammatory medications, gastrointestinal medicine within the past 3 months; and with regular intake of probiotics within the past 3 months. Participants who are incapable of living independently based on activities of daily living and instrumental activities of daily living score were excluded. A total of 107 candidates entered for screening and a total of 63 subjects enrolled for the study. This study was approved and monitored by the Institutional Review Board of Seoul National University (IRB No. 1801/002-015) and Bundang Jesaeng Hospital (IRB No. IMCN18-01), and written informed consent was obtained from all participants.

Study Capsules

Participants were provided with either placebo or probiotics. For the probiotics, participants were asked to consume two capsules after the meal in the morning and evening, which made a total of four capsules (a total of 1×10^9 colony-forming unit of *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI in soybean oil) to be taken per day. For the placebo, each capsule contained 500 mg of soybean oil only. Treatment products were not able to distinguish by package, color, taste, and smell in order to maintain treatment allocation concealed from participants and study staff. Test products were provided by Bifido Inc. (Seoul, Republic of Korea).

Randomization

Study coordinator who was not involved in the study generated a random sequence using GraphPad Prism (version 6.05; GraphPad Software, San Diego, CA) and the random number was stratified by sex with 1:1 allocation. The allocation sequence was concealed from the researchers and details of the allocated group were given on color code containing the sequential number which was prepared by product provider. Independent study coordinator dispensed either placebo or probiotics capsules according to a computer-generated randomized sequence.

Blinding

All participants, study coordinators, and researchers were blinded throughout the entire study. The study was unblinded after all statistical analyses were completed.

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Sample Collection

Twelve-hour fasting blood samples and stool samples were collected at each visit (baseline, 4th, 8th, and 12th week). Blood samples were collected into serum separating tube and ethylenediaminetetraacetic acid-coated tubes for serum and plasma isolation, respectively. Serum and plasma samples were aliquoted and immediately stored at −80°C for later analysis. For stool sample collection, we provided a stool collection tube that contains DNA stabilizing preservative reagent (Norgen Biotek, Thorold, ON, Canada). We instructed participants, following manufacturer's instructions, to collect fecal samples into the tubes and mix gently until the stool sample is completely submerged into the preservative. Participants were instructed to collect stool samples within the 48-hour period before visiting; the tubes were kept tightly sealed and stored at room temperature (15–25°C) until they were shipped. After the samples were shipped to the laboratory, aliquots of 180~200 mg of stool samples were immediately stored at −80°C until later analysis.

Outcome Assessments

The primary outcomes include results from cognitive function and mood tests at the end of the experiment. The secondary outcomes were gut microbial composition and anthropometric assessments measured at each visit; and neuronal biochemistry marker from the blood (brain-derived neurotrophic factor [BDNF]) at the end of the experiment.

Anthropometric measures

Body weight and height were measured at each visit using weight scales and stadiometers. BMI was calculated as weight in kilograms divided by height in meters squared.

Evaluation of intestinal health

Participants completed a general health questionnaire that asks about improvements in bowel habits at 4th, 8th, and 12th week, respectively. The questionnaire measures 10 bowel habits, asking whether there were improvements in the following parameters in the last 4 weeks: overall bowel health; frequency of defecation; amount of defecation; feeling of incomplete evacuation; stool odor; abdominal cramping; bowel sounds; number of gas passage; abdominal distention; and frequency of diarrhea. Participants responded with a 5-point scale that ranges from 1 ("not at all") to 5 ("very much"); and the higher scores indicate that there was improvement in each parameter.

Gut microbiota analysis

Genomic DNA extraction

Total bacterial DNA was isolated from stool by using the QIAamp fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with the following additional steps. Extracted genomic DNA was confirmed via gel electrophoresis and was quantified by spectrophotometer NanoDrop ND-2000 (Thermo Scientific, Waltham, MA).

Amplification of 16S rRNA gene and sequencing

Hypervariable regions (V3-V4) of 16S ribosomal ribonucleic acid (rRNA) gene were amplified using barcoded universal primers for each sample. Polymerase chain reaction (PCR) was carried out by using BioFact F-Star taq DNA polymerase (BioFACT, Seoul, Republic of Korea). Briefly, a final volume of 50 μL of PCR reaction contained about 20 ng of DNA template, 5 μL of 10× Taq buffer (20 mM Mg^{2+}), 1 μL of 10 mM dNTP mix, 2 μL of forward and reverse barcoded primers (10 pmol/μL), and 0.25 μL of DNA polymerase. PCR reactions were amplified using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The PCR program was as follows: initial for 5 minutes hold at 94°C, followed by 28 cycles of denaturation (30 seconds, 95°C), annealing (30 seconds, 60°C), and extension (30 seconds, 72°C), with a final extension step (10 minutes, 72°C) followed by holding at 4°C. The PCR product was confirmed by using 1% agarose gel electrophoresis and visualized under a Gel Doc system (BioRad, Hercules, CA). The amplified products were purified with PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Carlsbad, CA) and quantified by the Qubit 2.0 fluorometer (Invitrogen). The size of library was assessed by BioAnalyzer (Agilent Technologies, Santa Clara, CA). The amplicons were pooled and sequenced with an Illumina MiSeq sequencing system (Illumina, San Diego, CA).

Bioinformatic analysis of sequencing data

Microbial sequences were processed using QIIME2 (version 2019.1) (23). Briefly, sequences were denoised to remove the sequences with low-quality score and chimeras via DADA2. Then, denoised sequences were clustered into operational taxonomic units (OTUs) and OTU representative sequences were aligned based on SILVA database (version 132) at 99% sequence identity with scikit-learn Naive Bayes-based machine-learning classifier. A phylogenetic tree was generated using MAFFT and FastTree method for diversity analyses. Downstream analyses on alpha diversity were carried out to measure dissimilarities in richness and evenness of microbial community. Comparisons of relative abundance between groups were performed to identify the differential features across the samples.

Evaluation of cognitive function and mood status

The Korean version of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD-K) was used to measure cognitive function. The CERAD-K, a validated measure for the screening of Alzheimer's disease, assesses cognitive function including 11 tests measuring domains of language function, memory function, visuospatial processing function, and attention and executive function (24).

A validated 20-item self-reported questionnaire was used to ask the level of stress in a category of burn-out, depression, and anger during the past 1 month (25). Participants responded with a 5-point scale that ranges from 1 ("never") to 5 ("very often"). Total scores were calculated, and higher scores mean higher level of stress. The quality of life (QoL) was measured with the Satisfaction With Life Scale (SWLS), a validated subjected report of global life satisfaction (26). It consists of five items with a 7-point scale that ranges from 1 ("not at all") to 7 ("very much"). Responses were summed and higher scores indicate higher QoL. The Korean version of Geriatric Depression Scale (GDS-K) was used to evaluate the level of depression. The GDS-K is a 30-item self-reported questionnaire which is a validated instrument for the diagnosis of clinical depression (27). Each question was answered with binary responses ("yes" or "no") and scored as either 0 or 1 point. The cumulative score is calculated, and the higher score means the higher level of depression. The Positive Affect and Negative Affect Schedule (PANAS) is a validated self-report instrument in the assessment of positive and negative affect (28). The PANAS is comprised of two 10-item scales which

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measure both positive and negative affect, respectively. Each item is assessed with 5-point scale of 1 ("not at all") to 5 ("very much"). The summed scores from each positive and negative affect indicate the level positive and negative affect, respectively.

Serum biochemical markers

Serum BDNF level was measured using BDNF DuoSet ELISA kit (DY248; R&D Systems, Wiesbaden-Nordenstadt, Germany) and DuoSet Ancillary Reagent kit 2 (DY008; R&D Systems) according to the manufacturer's instructions. Briefly, sample or standard was added to a plate coated with capture antibody and the plate was incubated for 2 hours at room temperature. After washing the plate sufficiently, detection antibody was added to the plate and the plate was incubated for 2 hours at room temperature. Streptavidin conjugated to horseradish peroxidase was added to each well and the plate was incubated for 20 minutes at room temperature. Then substrate solution was added to each well for 20 minutes of incubation at room temperature following sufficient washing with wash buffer, and the plate was ready for determining the optical density at 450 nm wavelength using a microplate reader (SpectraMax iD3, Molecular Devices, Austria).

Statistical Analysis

Sample size

To detect a significant change in cognitive function with a two-sided 5% significance level and a power of 80%, a sample size of 32 was determined, given a 20% of dropout rate.

Analysis plan

The normality assumption and homogeneity of variance were tested by Kolmogrov–Smrinov test for study variables. For the comparison analysis of variables at the baseline between groups, we used independent *t*-test, χ^2 tests, or Fisher's Exact Tests. To compare the difference between groups at each time point and delta value between the visits (Δ(Week 4–Week 0), Δ(Week 8–Week 0), and Δ(Week 12–Week 0)) between the two groups, we performed unpaired *t*-test, Mann–Whitney *U* test or generalized linear model (GLM). To compare the difference between baseline and the data from end point (Week 12), we used paired *t*-test or Wilcoxon signed rank test. To detect the difference between groups over the visits, we used a mixed-model analysis of variance (ANOVA) or Friedman test as a nonparametric alternative to the repeated measures ANOVA. Correlations were assessed by Spearman rank correlation analysis. Correction for multiple testing was performed based on the false discovery rate or Bonferroni correction. The $p < .05$ and false discovery rate <0.05 were considered statistically significant in all statistical analyses. All statistical analyses were conducted using Partek (version 6.6; Partek, Saint Louis, MI), SPSS (version 25.0; SPSS Inc., Chicago, IL), or GraphPad Prism (version 6.0; GraphPad Software, San Diego, CA).

Results

General Characteristics of Participants at Baseline

A total of 107 volunteers were screened for eligibility and 63 subjects were enrolled for the study (Supplementary Figure 1). Sixty-three participants were randomized, with 31 and 32 subjects in the placebo and probiotics group, respectively. Fifty-three individuals completed the study and 10 participants withdrew the consent and discontinued the study, and no clinically relevant adverse events were reported during the intervention. When comparing characteristics of participants who withdrew ($N = 10$) and those who completed the trial ($N = 53$), there were no significant differences (Supplementary Table 1). Therefore, we confirmed that randomization was successful. In all analyses, we included data from participants who completed the intervention. Demographic and clinical characteristics at baseline are summarized in Supplementary Table 2. Average age was 72.00 and 71.11 years in the placebo and probiotics group, respectively, with no significant difference ($p = .4538$). The ratio of male to female and BMI did not significantly differ between two groups. Socioeconomic characteristics, including educational level, marital status and type of household, and other health-related characteristics such as cigarette use, alcohol use, physical activity, and self-evaluated health status, were not different between the placebo and probiotics group. In addition, cognitive functions and depression scores, determined by MMSE and Geriatric Depression Scale (GDS-K), were not different between two groups at the baseline.

Probiotic Supplementation Beneficially Influenced Intestinal Health and Gut Microbial Communities

To assess the effect of probiotics on intestinal health, participants filled questionnaire at 4th week, 8th week, and 12th week, respectively, asking whether there were improvements in bowel habits in the last 4 weeks. Bowel habits such as frequency and amount of defecation; feeling of incomplete evacuation; stool odor; number of gas passage; bowel sounds; and abdominal distention were not significantly improved both in the placebo and probiotics group during the intervention period (data not shown); however, scores in frequency of gas passage and abdominal distention showed significant improvements in the probiotics group compared with the placebo (3.44 ± 0.19 vs 2.77 ± 0.21; 3.15 ± 0.22 vs 2.46 ± 0.22, respectively; $p < .05$, Figure 1A and B).

In order to address whether the improvement was driven by any changes in the intestinal bacterial communities, gut microbiome profiling analysis was performed in all participants. Bacterial genomic DNAs from stool samples collected at baseline, 4th week, 8th week, and 12th week were sequenced using 16S rRNA sequencing technology. After preprocessing of bacterial sequences for quality control as described in the Methods, we obtained a total of 10,273,269 raw reads and average reads of 80,260 per sample. To examine the effect of probiotics consumption on gut microbial diversity, we calculated Pielou's evenness index, Faith's phylogenetic diversity,

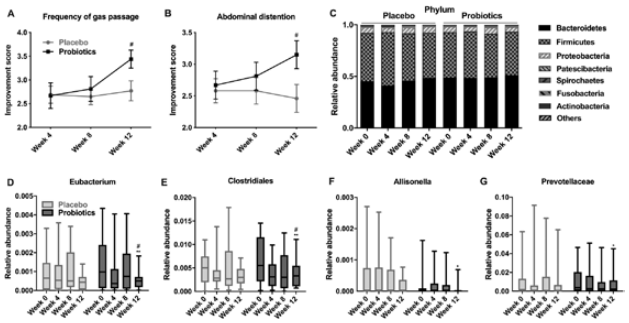

Figure 1. Beneficial influence of probiotic supplementation on intestinal health and gut microbiota. (**A,B**) Improvement scores in frequency of gas passage and abdominal distention measured at each visit are shown. Data are presented as mean (SEM). (**C**) Relative abundance of the gut microbiota at the phylum level and (**D–G**) at the genus level was measured throughout the intervention. Data are presented as mean (min-max). $^{\#}p < .05$ based on the Mann–Whitney *U* test; $^{**}p < .005$, $^{*}p < .05$ based on a post hoc analysis of Friedman test.

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observed OTUs, and Shannon's diversity index. We found no significant changes in the diversity both in the placebo and probiotics group during the intervention period (Supplementary Figure 2A–D). Further, we compared the relative abundance of OTUs and specific bacterial taxa at the different phylogenetic levels. Microbial composition at OTU level showed no significant differences during the intervention both in the placebo and probiotics group (Supplementary Figure 2E). At the phylum level, no significant changes in relative abundance were detected during the intervention both in the placebo and probiotics group (Figure 1C). However, at the genus level, we found significant changes in the gut microbial composition in the probiotics group and no changes in the control group (Figure 1D–G). The relative abundances of *Eubacterium, Allisonella,* Clostridiales, and Prevotellaceae gradually changed during the intervention, and significantly decreased at Week 12 in the probiotics group ($p < .05$).

Probiotic Supplementation Improved Brain Function and Increased Peripheral BDNF Levels

To evaluate the impacts of probiotic supplementation on cognitive function, each participant was tested by the CERAD-K, a validated cognitive test battery that scores language, memory, visuo-spatial processing, and attention/executive functions. The assessment was performed at baseline, Week 4, and Week 12 (Table 1). The changes at the fourth week from baseline in the probiotics group were not different from those in the placebo group for all the domains of the cognitive assessment; however, the changes at Week 12 from baseline in the scores of mental flexibility test were significantly different between placebo and probiotics group (Table 2). Interestingly, mental flexibility showed a significant improvement at Week 12 in the probiotics group compared with the placebo group ($p < .05$, Figure 2A). In addition, study subjects filled series of questionnaires to evaluate the impact of probiotics on mood status including quality of life, stress, depression, and positive and negative affect. The 12-week consumption of probiotics did not change the scores of quality of life, GDS-K, and PANAS; however, it did affect the stress score (Table 2). While the stress score was increased in the placebo group (1.38 ± 0.86), it was dramatically decreased in the probiotics group (−2.85 ± 1.16; $p < .05$, Figure 2B).

The observations that probiotic supplementation improved the cognitive function and mental stress prompted us to determine the level of BDNF in blood. BDNF is a neurotrophic factor known to be crucial for learning, memory function, and stress. In contrast to the placebo group (−3.32 ± 2.35), serum BDNF level was significantly increased at Week 12 in the probiotics group (3.68 ± 2.69; $p < .05$, Figure 3A). In addition, to address the question of whether changes in intestinal bacterial communities be related to the serum level of BDNF, we conducted correlation analysis between the relative abundance of each genera and the level of BDNF. It is of interest that *Eubacterium* and Clostridiales showed a significant negative correlation with the level of serum BDNF only in the probiotics group ($R_S = -0.37$ and $R_S = -0.39$; $p < .05$, Figure 3B). These findings suggest that reduction in the relative abundances of *Eubacterium* and Clostridiales in the gut driven by probiotic supplementation closely related to the increase in the serum BDNF, thereby improving brain functions.

Discussion

In the present study, we conducted a randomized, double-blind, placebo-controlled, multicenter trial to address the impact of

Table 1. Cognitive Function Before and After the Intervention

	Placebo ($N = 26$)				Probiotics ($N = 27$)					
	Week 0	Week 4	Week 12	[a]*p*-value	Week 0	Week 4	Week 12	[a]*p*-value	[b]*p* for Δ(Week 4–Week 0)	[b]*p* for Δ(Week 12–Week 0)
Language function										
Verbal fluency	14.96 (4.05)	16.42 (4.59)*	16.88 (4.55)	.01	14.44 (4.48)	15.67 (5.17)	15.41 (4.17)	.40	.86	.39
Boston naming test	11.69 (2.19)	12.19 (2.26)	12.23 (2.23)	.28	12.15 (1.56)	12.70 1.49)	12.96 (1.34)**	<.005	.70	.23
Memory function										
Word list encoding	18.92 (4.42)	20.77 (3.63)	22.23 (4.74)**	<.001	18.26 (2.81)	21.33 (3.10)**	22.22 (3.79)**	<.001	.12	.47
Word list recall	6.38 (1.92)	7.27 (1.78)	7.54 (1.92)*	.01	6.19 (1.82)	6.85 (1.88)	7.52 (1.65)**	<.005	.58	.68
Word list savings	83.83 (18.87)	94.24 (14.47)	92.21 (16.50)	.13	84.68 (23.06)	83.44 (19.30)†	89.30 (13.27)	.18	.15	.71
Word list recognition	8.77 (1.77)	9.27 (1.25)	9.23 (1.53)	.09	9.22 (0.97)	9.37 (0.74)	9.63 (0.63)	.30	.38	.88
Constructional praxis recall	8.15 (2.81)	8.88 (2.57)	9.23 (2.30)	.02	7.93 (3.05)	8.89 (2.39)	9.52 (1.72)*	<.001	.90	.42
Visuo-spatial processing function										
Constructional praxis	10.04 (1.43)	10.00 (1.60)	10.27 (1.34)	.36	10.00 (1.64)	10.33 (1.44)	10.52 (1.01)	.31	.55	.74
Attention and executive function										
Trail making test A	61.88 (26.82)	49.73 (17.99)*	47.35 (15.91)**	<.001	47.33 (18.78)†	46.22 (21.47)	44.41 (21.62)	.06	.36	.21
Trail making test B	189.69 (82.28)	164.65 (77.22)	161.19 (78.26)	.15	172.59 (86.95)	148.26 (67.32)	131.11 (58.44)*	.01	.97	.39
Mental flexibility	2.15 (0.97)	2.46 (1.54)	2.52 (1.68)	.48	2.72 (1.56)	2.30 (0.96)	2.08 (0.85)	.10	.06	.03
Digit span test	13.35 (5.18)	14.23 (4.23)	13.65 (4.54)	.59	13.41 (4.48)	15.33 (3.81)**	14.59 (3.88)*	<.005	.23	.16

Note: Data are presented as mean (*SD*).
[a]*p*-value from Friedman test; [b]*p*-value from the Mann–Whitney *U* test or GLM analysis (after adjusting for baseline value) for between-group analysis for the comparison of delta value; $^{**}p < .005$, $^{*}p < .05$ based on a post hoc analysis of Friedman test; $^{\dagger}p < .05$ based on the Mann–Whitney *U* test for between-group analysis at each time point.

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Table 2. Mood Status Before and After the Intervention

	Placebo (N = 26)				Probiotics (N = 27)					
	Week 0	Week 4	Week 12	[a]p-value	Week 0	Week 4	Week 12	[a]p-value	[b]p for Δ(Week 4–Week 0)	[b]p for Δ(Week 12–Week 0)
QoL	22.88 (7.26)	21.38 (5.45)	21.58 (6.68)	.07	20.74 (5.85)	20.33 (6.77)	20.89 (6.93)	.38	.12	.18
Stress	31.12 (12.24)	31.73 (13.17)	32.50 (11.46)	.37	31.74 (11.77)	29.33 (8.61)	28.89 (7.77)	.10	.10	.04
Depression	6.31 (5.87)	6.23 (6.16)	6.15 (5.72)	.74	7.59 (4.61)	6.96 (5.36)	6.41 (5.35)	.12	.27	.39
Positive affect	28.46 (7.89)	26.08 (7.20)*	25.58 (6.25)*	.09	28.59 (6.69)	27.52 (6.35)	28.41 (8.28)	.86	.59	.12
Negative affect	16.62 (6.54)	15.54 (5.41)	15.54 (5.27)	.39	14.78 (4.73)	14.67 (4.54)	14.04 (4.75)	.66	.88	.69

Note: Data are presented as mean (*SD*). QoL = Quality of life.
[a]p-value from Friedman test; [b]p-value from the Mann–Whitney U test for between-group analysis for the comparison of delta; $^{**}p < .005$, $^{*}p < .05$ based on a post hoc analysis of Friedman test; $^{\dagger}p < .05$ based on the Mann–Whitney U test for between-group analysis at each time point.

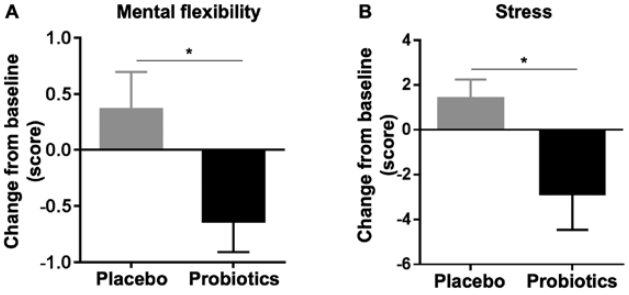

Figure 2. Improved cognitive and mental functioning after probiotic supplementation. (**A**) Change from baseline of cognitive performance score in the mental flexibility test is shown. Reduction in the performance score of mental flexibility indicates improved attention and executive function. (**B**) Change from baseline of stress level is shown. Reduction in the change indicates a reduced level of mental stress. Data are presented as mean (SEM). $^{*}p < .05$ based on the Mann–Whitney U test for the comparison analysis of changes from baseline between the two groups.

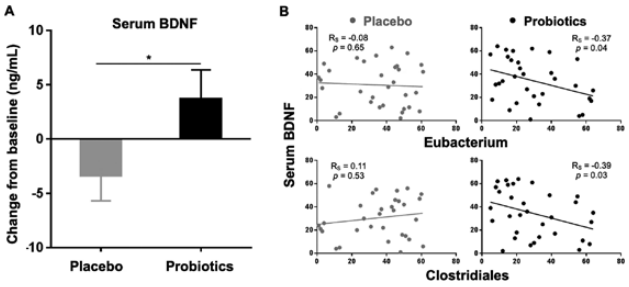

Figure 3. Elevated level of serum BDNF after probiotic supplementation. (**A**) Change from baseline of serum BDNF is shown. Data are presented as mean (SEM). $^{*}p < .05$ for time × treatment from a mixed-model analysis of variance. (**B**) Scatter diagrams with regression lines show the relationship between relative abundance of shifted gut bacteria after probiotic supplementation and the level of serum BDNF. Measurements were rank-normalized and plotted separately for the placebo and probiotics group. Correlation coefficient (R_s) and p-values based on Spearman rank correlation analysis. BDNF = Brain-derived neurotrophic factor.

probiotics on intestinal health and how they contribute to ameliorating cognitive and mental decline in the older adults. Our findings demonstrate that probiotics have system-wide effects on the gut–brain axis in healthy community-dwelling older adults by promoting cognitive and mental health and changing the gut microbial composition.

Emerging evidence has suggested that probiotics have considerable impacts on various cerebral functions through the regulation of the gut–brain axis, but the current studies are mainly focused on patients with mild cognitive impairment, Alzheimer's disease, and major depressive disorder (29–32). However, while neurodegenerative disorders and psychological distress are a common threat to well-being in old age, nutritional intervention to prevent or delay age-associated decline in brain function in the general older population is still underexplored. In fact, there is only one report on the effects of probiotic consumption in healthy older adults, showing that milk fermented by *Lactobacillus helveticus* IDCC3801 improved cognitive functions in healthy older adults (33). However, the sample size was too small and the criteria for the study participants did not represent the general population of older adults. Therefore, the critical need for clinical studies in the general population has been raised. To the best of our knowledge, this is the first well-controlled

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clinical study demonstrating system-wide effects of probiotics on the gut–brain axis, which encompasses the large-scale analysis of the gut microbiota and multiple aspects of brain functions in healthy older population.

Randomized controlled trials (RCTs) are very challenging for several issues but the most rigorous method, which provides the most reliable evidence for clinical practice; however, there are few RCTs specifically designed for older adults because it is difficult to recruit older people, particularly community-dwelling older adults (34–36). Therefore, older adults have been excluded from clinical trials and most studies focused on older group of patients (36). In addition, it is relatively hard to follow up and contact older people during a trial which increases the risk of dropout and reduces compliance (36,37). Despite these challenges, in the present study, participant compliance was good as the average rate of compliance to intervention was 96.5%, with a dropout rate of 15.9% only. Moreover, it is important to note that the present study recruited older adults without diseases, not focusing on good responders to a treatment effect such as patients with neurological disorders, which makes our findings more applicable as a generalized health care strategy in community-dwelling older population.

In the probiotics group, the gut microbial composition shifted gradually, and the most relevant change was the reduction in the abundance of bacteria that cause inflammation including *Eubacterium*, *Allisonella*, and Prevotellaceae. It has been identified that *Eubacterium* and Prevotellaceae species, which were significantly reduced after probiotic consumption, are proinflammatory microbiota associated with autoimmune disease and chronic intestinal inflammation in mice (38,39). Of note, the genus *Allisonella*, whose abundance was significantly reduced in the probiotics group, produces histamine, a biogenic monoamine inducing proinflammatory response both centrally and systemically (40). Moreover, in patients with Alzheimer's disease, elevated levels of histamine stimulate neuroinflammation via induction of low-grade systemic inflammation (40). Therefore, these findings may parallel our hypothesis that probiotic supplementation in the older adults may negatively affect inflammaging, a characteristic of chronic low-grade inflammatory status in older adults, via the modulation of microbial composition. However, further studies are required to assess whether the probiotic supplementation affects immunological mechanisms.

The findings of the present study suggest that interaction between the gut microbiota and the central nervous system may underlie the improvements in cognitive and cerebral functioning upon probiotic supplementation and explain the concomitant changes in peripheral neuromodulators. BDNF, a neurotrophic factor vital for synaptic formation, plasticity, and neuroimmune responses, has long been studied to assess its critical role in learning, memory formation, and affective disorders (41,42). Previously, the influence of diet and nutrition on BDNF has been explored; and serum BDNF has been shown to be increased in response to dietary supplements in humans. For example, a 1-week of oral consumption of α-linolenic acid increased the level of serum BDNF in healthy young adults (43). Also, a 6-week supplementation with natural extracts rich in flavonoids and polyphenolic compounds enhanced serum BDNF levels in physically active men (44). In the present study, it was notable that the beneficial impact of a 12-week probiotic intervention on serum BDNF levels was evident in older population. More recently, BDNF has emerged as a pivotal link in the gut–brain axis (41,42). Several studies demonstrated that gut dysbiosis correlates with reduced expression of BDNF, which alters cognitive function and triggers anxiety-like behavior in germ-free animals (45,46), supporting a role of BDNF in the gut–brain axis. Interestingly, we observed that the relative abundance of significantly shifted gut microbes correlated with the level of serum BDNF in the probiotics group only. This indicates that administration of probiotics may affect the interaction between the gut microbiome and the host BDNF, thereby improving brain functions. Overall, the evidence from this study shows that the shifts in microbial community mirrored changes in the cognitive and mental scores.

Several mechanisms could explain the interaction between changes in abundance of commensal bacteria and brain function observed in the probiotics group. First, it is plausible that the production of neurotransmitters, such as γ-Aminobutyric acid (GABA), dopamine, acetylcholine, serotonin, by commensal bacteria, and neurochemicals including BDNF, may directly or indirectly modulate cognition and mood status (47). As shown in a previous study, probiotic administration influences GABA receptor throughout the brain, with reduced stress-induced anxiety- and depression-like behaviors in rodents (48). Moreover, inflammation-mediated pathways might initiate the pathogenesis of neurodegeneration via the microbiota–gut–brain axis. With respect to inflammaging during normal aging, chronic low-grade inflammation in older adults may affect neuroinflammation by modulating glial cells, which stimulates cognitive impairment (47). One of the routes to translate systemic inflammatory signals into the brain is stimulation of microglia by peripheral cytokines that cross the blood–brain barrier, leading to a proinflammatory status in the brain and dysregulation of neurological processes (47). Additionally, the immunomodulatory roles of circulating immune cells in neuroplasticity also affect the expression of BDNF (49,50). Therefore, it is plausible that mitigation of inflammaging in older adults with probiotic intervention might positively impact on cognitive and mental functions via the modulation of BDNF signaling. Further studies are needed to clearly demonstrate the effect of probiotics on inflammatory status and gut microbiome at the functional level.

The present study is not without limitations. First, direct evidence of improvement in peripheral and cerebral inflammation by probiotic consumption is lacking, which might be the crucial interface linking the gut–brain axis in the present study. Therefore, further mechanistic studies might be needed to elucidate the role of probiotic supplementation by finding biomarkers to link the axis. Second, although cognitive functions were evaluated by validated neuropsychological assessment battery tasks taking at least 60 minutes per subject by a professionally trained panel, psychological assessments of mood status were based on the participants' self-reporting; therefore, possible recall bias may exist. Additionally, although our results indicated that the benefit of 3 months duration of probiotic intervention was evident, there were no significant changes in some of the cognitive functions in the neuropsychological assessment battery in which we assume that the study duration was not enough to monitor the improvements. Therefore, further studies are required with a longer period of intervention. Despite these limitations, this is the first study examining the effects of probiotic supplementation on brain functions in community-dwelling older adults. In conclusion, our study showed that probiotic supplementation is beneficial for improving cognitive and mental health in community-dwelling healthy older adults with changes in gut microbial composition. These results provide evidence that probiotics have health-promoting properties as part of a healthy diet in the general population of independently living older adults.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Authors' Contributions

C.K., H.B., and D.S. designed the research; C.K., L.C., and M.S. conducted the research; C.K. performed bioinformatic and statistical analyses and analyzed the data; W.C. conducted the statistical analysis; and C.K. and D.S. wrote the manuscript. The authors thank S.J. for his assistance in recruitment of participants; D.S. had primary responsibility for final content. All authors read and approved the final manuscript.

Conflict of Interest

None reported.

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The long-term effects of probiotics in the therapy of ulcerative colitis: A clinical study

Vincenzo Davide Palumbo[a,b], Marcello Romeo[c], Antonella Marino Gammazza[a,c], Francesco Carini[c,d], Provvidenza Damiani[d], Giuseppe Damiano[c], Salvatore Buscemi[c], Attilio Ignazio Lo Monte[c,d], Alice Gerges-Geagea[c,e], Abdo Jurjus[e], Giovanni Tomasello[a,c,d]

Aim. Intestinal dysbiosis seems to be the leading cause of inflammatory bowel diseases, and probiotics seems to represent the proper support against their occurrence. Actually, probiotic blends and anti-inflammatory drugs represent a weapon against inflammatory bowel diseases. The present study evaluates the long-term (2 years) effects of combination therapy (mesalazine plus a probiotic blend of Lactobacillus salivarius, Lactobacillus acidophilus and Bifidobacterium bifidus strain BGN4) on ulcerative colitis activity.
Method. Sixty patients with moderate-to-severe ulcerative colitis were enrolled: 30 of them were treated with a single daily oral administration of mesalazine 1200 mg; 30 patients received a single daily oral administration of mesalazine 1200 mg and a double daily administration of a probiotic blend of Lactobacillus salivarius, Lactobacillus acidophilus and Bifidobacterium bifidus strain BGN4. The treatment was carried out for two years and the clinical response evaluated according to the Modified Mayo Disease Activity Index.
Results. All patients treated with combination therapy showed better improvement compared to the controls. In particular, the beneficial effects of probiotics were evident even after two years of treatment.
Conclusions. A long-term treatment modality of anti-inflammatory drugs and probiotics is viable and could be an alternative to corticosteroids in mild-to moderate ulcerative colitis.

Key words: bifidobacteria, inflammatory bowel diseases, lactobacilli, microbiota, ulcerative colitis

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[a]Euro-Mediterranean Institute of Science and Technology (IEMEST), Palermo, Italy
[b]Department of Surgical, Oncological and Stomatological Disciplines, School of Medicine, University of Palermo, Palermo, Italy
[c]Department of Experimental Biomedicine and Clinical Neuroscience, School of Medicine, University of Palermo, Palermo, Italy
[d]P. Giaccone University Hospital, Palermo, Italy
[e]Department of Anatomy, Cell Biology and Physiology, School of Medidcine, Aamerican University of Beirut, Beirut, Lebanon
Corresponding author: Vincenzo Davide Palumbo, e-mail: vincenzopalumbo@iemest.eu

INTRODUCTION

Inflammatory bowel disease worldwide incidence and prevalence have been increasing in the last few decades. Ulcerative colitis (UC) is one of the two major types of IBD, along with Crohn disease (CD). Unlike CD, which can affect any part of the gastrointestinal tract, UC characteristically involves the large bowel[1,2].

There is a large debate about the exact aetiology of UC. Proposed causes include environmental factors, immune dysfunction, and a likely genetic predisposition. The current hypothesis is that genetically susceptible individuals have abnormalities of humoral and cell-mediated immunity and a generalized enhanced reactivity against commensal intestinal bacteria[3]. This dysregulated mucosal immune response predisposes to colonic inflammation[3]. Whether these abnormalities are the cause or the result of the intense systemic inflammatory response in UC is still unresolved. However, it is well documented that bacterial microflora is altered in patients with active disease[4]. Recent studies reported a great variation in the effects of microbiota, focusing, in particular, on the effects of a pro-inflammatory enterotype on mucosal layer and disease activity[5].

Modulation of the intestinal microbiota can be performed either by antibiotics or by probiotics, but the former are not good candidates for chronic disease because of antibiotic resistance, potential side effects, and ecological concerns[6]. Therefore, the use of probiotics in IBD could be considered a potential aid to the current conventional therapies. An accurate analysis of scientific data proves that the efficacy of probiotics in the treatment of various diseases has been amply demonstrated and confirmed[7-9]. Actually, several studies have focused on the effects of probiotic blends on enteral microbiota, especially in those cases of dysbiosis, when the normal concentration of "good" bacterial flora is impaired by the presence of pathogenic bacteria[10,11].

Currently, the standard treatment of UC relies on an initial medical management with corticosteroids and anti-inflammatory agents, such as mesalazine, in conjunction with a symptomatic treatment with antidiarrheal agents and rehydration. These treatments have been proven not to be always reliable in controlling the clinical course of

Table 1. Modified Mayo Disease Activity Index.

Grade	Bowel frequency	Rectal bleeding	Physician's global assessment	Endoscopy/sigmoidoscopy finding
0	Normal number of stools per day for this patient	No blood seen	Normal	Normal or inactive disease
1	1 or 2 more stools than normal	Streaks of blood with stool less than half the time	Mild disease	Mild disease (erythema, decreased vascular pattern)
2	3 or 4 more stools than normal	Obvious blood with stool most of the time	Moderate disease	Moderate disease (marked erythema, absent vascular pattern, friability, erosions)
3	5 or more stools than normal	Blood alone passed	Severe disease	Severe disease (spontaneous bleeding, ulceration)

the disease[12,13] and present side effects in a significant proportion of patients who do not tolerate the existing treatments[14]. In the present open-labeled randomized controlled study, standard corticosteroid treatment was substituted for a combination therapy (anti-inflammatory + probiotics) for two years, in moderate-to-severe UC and the disease activity was periodically followed-up according to the Modified Mayo Disease Activity Index (MMDAI) (ref.[15]).

Table 2. Modified Mayo Disease Activity Index (MMDAI) from t_0 to t_4, in group A, B and A vs B.

MMDAI	A	B	A vs B
t_0	10.1±1.4	10.2±1.6	ns
t_1	7.2±2.0	6.0±1.5	$P = 0.0109$
t_2	5.4±1.3	4.8±0.7	$P = 0.0232$
t_3	5.7±1.4	4.8±0.8	$P = 0.0035$
t_4	6.1±2.0	4.4±0.8	$P = 0.0001$

Table 3. Physician's global assessment from t_0 to t_4, in group A, B and A vs B.

Physician's global assessment	A	B	A vs B
t_0	2.6±0.7	2.7±0.5	ns
t_1	1.9±0.7	1.7±0.7	ns
t_2	1.5±0.6	1.3±0.5	ns
t_3	1.4±0.6	1.2±0.4	ns
t_4	1.5±0.6	1.2±0.4	$P = 0.0040$

MATERIALS AND METHODS

From January 2011 to December 2012, 60 UC patients were evaluated clinically and endoscopically to establish their disease activity, in accordance with the MMDAI (ref.[15]), a simplified composite score incorporating four variables: stool frequency, rectal bleeding, mucosal appearance and physician's rating of disease activity (Table 1). By employing a four point scoring scale for each variable, the relative simplicity of the index reduces the impact of physician and patient subjectivity in disease scoring. Interestingly, the stool frequency score is not an absolute number, but relative to "normal" for that subject. The MMDAI was modified by the deletion of "friability" from an endoscopy score equal to 1; in fact, the assessment of mucosa "friability" is an important subjective parameter and its deletion contributes to the objectivity of the chosen score. Patients over 18 years of age with UC and a moderate-to-severe disease (activity index: 8-12) were considered eligible for the study. The diagnosis of UC, was established on the basis of standard clinical, endoscopic and histological criteria. All subjects were out-patients, attending our Gastroenterology Unit. A condition of steroid dependence, renal impairment, pregnancy, lactation or established low compliance, was considered as an exclusion criterium. The use of other drugs, such as rectal mesalazine or steroid preparations, was not allowed during investigation. Patients were free to leave the study at any time (withdrawal of consent). Other reasons for withdrawal from the investigation were: lack of adherence to the therapeutic schedule or programmed controls (< 85%, poor compliance); onset of symptoms of relapse, confirmed by instrumental procedures (therapeutic failure); onset of drug-related adverse events requiring interruption of treatment. The study was approved by the local ethics committee and all participants subscribed to an informed consent.

The patients were divided into two homogeneous groups: group A, including 22 male and 8 female subjects aged 35-69 years (mean 43 years), was treated pharmacologically with 1200 mg of oral mesalazine once-daily (Mesavancol® 1200 mg cpr, Giuliani spa, Milan); group B, which included 19 male and 11 female UC patients aged between 28 and 71 years (mean 46 years), was treated with a single daily administration of oral mesalazine 1200 mg (Mesavancol® 1200 mg cpr, Giuliani spa, Milan) and a double administration of a probiotic blend of Lactobacillus salivarius, Lactobacillus acidophilus and Bifidobacterium bifidus strain BGN4 (Acronelle®, Bromatech srl, Milan, Italy). The treatment was carried out in both groups for two years.

Considering as "t_0" the first evaluation, all patients were subsequently followed-up for 6 (t_1), 12 (t_2), 18 (t_3) and 24 (t_4) months. A new reassessment of the activity score was carried out at every check-point. Data were evaluated statistically using the ANOVA method for re-

peated measures (ANOVA Repeated Measures), and the two groups compared by means of t test (unpaired t test).

RESULTS

During the 24 months of study, patients treated with mesalazine and probiotic blend showed better results than those reached by patients treated with mesalazine alone. In particular, as Table 2 suggests, the benefits of the probiotic blend in combination with the anti-inflammatory treatment are tangible and statistically significant after a period of at least 18 months. Both groups showed an effective improvement of patient general clinical condition which is reflected by a global improvement of their MMDAI, but group B patients advantaged from the use of probiotics which, probably, enhanced the effects of the anti-inflammatory treatment.

Table 3 underlines the positive effects of single daily administration of mesalazine in UC patients. Interestingly, in group B, the anti-inflammatory action of the treatment is evidently powered by probiotics, which ameliorate the clinical response and shorten significantly the time of recovery. Furthermore, the combined therapy contributed to maintain constantly low the score in group B, whose patients showed an acceptable overall clinical condition up to the end of the study. However, a slight deterioration could be observed over time, in group A. Data analysis during the 2 years of treatment in both groups, corroborates the showed results, remarking the slight benefit of the combination therapy compared to the sole use of mesalazine (Table 3).

Stool frequency, the second parameter considered, also showed a significant improvement due to the combined treatment, with a slight reduction of frequency in group B compared to group A (Table 4). Strangely, there was a slight deterioration at 18 months in group B, whereas in group A, even in this case, a moderate loss of effectiveness of the anti-inflammatory therapy could be recognized over time. Comparing the two groups, the reduction of stool frequency became statistically significant in group B, at 6 and 24 months of therapy. The table shows a small reversal of the trend at 18 months, when the patients of group B seemed to lose the advantage supplied by probiotics.

Considering the endoscopic picture (Table 5), both treatments were already effective only after 6 months. In group B, patients showed a significant improvement of intestinal mucosa aspect, compared to group A, and, as already remarked for the overall clinical condition, the beneficial effects of combination therapy remained constant for the entire period of study, compared to group A, whose patients lost some of those benefits already at t_3.

The evaluation of rectal bleeding more or less followed the same time lapse already seen for the endoscopic picture (Table 6). In fact, bleeding decreased in both groups already at t_1, but whereas group B enjoyed the positive effects of probiotics up to 2 years, showing a certain constancy of values at each check-point, group A, once again, got progressively worse from 18 months of treatment onwards.

Table 4. Stool frequency from t_0 to t_4, in group A, B and A vs B.

Stool frequency	A	B	A vs B
t_0	2.4±0.5	2.4±0.6	ns
t_1	1.8±0.6	1.4±0.5	$P = 0.0021$
t_2	1.3±0.5	1.2±0.4	ns
t_3	1.3±0.5	1.3±0.5	ns
t_4	1.5±0.6	1.0±0.2	$P = 0.0006$

Table 5. Endoscopic picture from t_0 to t_4, in group A, B and A vs B.

Endoscopic finding	A	B	A vs B
t_0	2.5±0.5	2.5±0.6	ns
t_1	1.8±0.8	1.5±0.6	ns
t_2	1.3±0.5	1.2±0.4	ns
t_3	1.6±0.5	1.1±0.3	$P = 0.0005$
t_4	1.6±0.6	1.1±0.3	$P = 0.0005$

Table 6. Rectal bleeding from t_0 to t_4, in group A, B and A vs B.

Rectal bleeding	A	B	A vs B
t_0	2.6±0.6	2.5±0.6	ns
t_1	1.7±0.7	1.4±0.5	$P = 0.0498$
t_2	1.3±0.4	1.1±0.3	ns
t_3	1.4±0.5	1.1±0.3	$P = 0.0374$
t_4	1.5±0.7	1.1±0.3	$P = 0.0024$

DISCUSSION

In the last few years, intestinal microbiota seems to be increasingly involved in UC pathogenesis[16,17]. The characteristic chronic inflammation of the colonic mucosa in UC is likely due to the constant exposure of the mucosal layer to antigenic endoluminal stimuli. Numerous studies have identified in intestinal dismicrobism the most important endoluminal antigenic stimulus; this, along with the change of the whole intestinal microenvironment, could hyperstimulate the immune system and trigger the inflammatory process[18-20].

Microbiological studies have detected a significant reduction of Bifidobacteria and Lactobacilli and an overgrowth of specific pathogenic strains, such as Deltaproteobacteria and Bilophila wadsworthia. The use of probiotics in combination with the standard treatment, improves patients' quality of life and life expectancy, reducing significantly clinical symptoms and minimizing side effects[21-25]. The results of our study confirm the beneficial effects of probiotics on UC activity, partly by improving patient's response to anti-inflammatory treatment. Comparing patients treated with probiotic blend and mesalazine, and those patients who received the anti-

inflammatory alone, data report a significant improvement of MMDAI in the former group, after 18 months. Interestingly, the association of probiotics with mesalazine, seems to guarantee a stable effect in the whole period of observation, different from the anti-inflammatory therapy alone, which seems to be burdened by an unsteady response. Oral 5-ASA administered once daily has been demonstrated to be as effective and safe as conventional dosing (twice or three times daily) for maintenance of remission in quiescent UC (ref.[26]). The choice of testing a specific probiotic blend (Acronelle®, Bromatech srl, Milan, Italy) was suggested by the distinguishing features of the bacterial strains contained in the considered probiotic blend: Lactobacillus salivarius has a high anti-inflammatory and antibacterial activity, especially against some of those pathogenic bacterial strains, usually present on the intestinal mucosa of IBD patients[27]; Bifidobacterium bifidum BGN 4 supports Lactobacilli against inflammation, thanks to the chiro-inositol present in its cell membrane, which seems to reduce the production of pro-inflammatory cytokines[28]. Although guidelines[29] suggest to treat moderate-to-severe UC with corticosteroids, the contemporary use of Mesavancol® and Acronelle® allowed to reach an encouraging result, avoiding all those therapy side effects which usually decrease patient compliance. For the first time, the long-term efficacy (2 years) of such a probiotic blend has been proven, also demonstrating the synergistic effect on disease activity, especially when coupled with anti-inflammatory treatment. Several authors have already contributed to research in this field, however, none of them has followed-up moderate-to-severe UC patients, for a such long period of time. Ishikawa et al. (ref.[30]) reported that Bifidobacteria-fermented milk (BFM) supplementation reduces the luminal butyrate concentration, a key molecule in the remission of colitis. This reduction reflects the increased uptake or oxidation of SCFAs by the improved colorectal mucosa. Similarly, Kato et al. (ref.[31]) found increased levels of faecal butyrate, propionate, and SCFA acid concentrations in patients with active UC (mild to moderate), who received BFM together with conventional treatment. In this pilot study, patients supplemented with BFM showed a significantly lower clinical activity index than the placebo group. Likewise, the post-treatment endoscopic index and histological score were reduced in the BFM group. Probio-Tec AB-25, a mixture of Lactobacillus acidophilus strain La-5 and Bifidobacterium animalis subsp. lactis strain Bb-12, was tested for the maintenance of remission in patients with left-sided UC, in a 1-year, prospective, randomized, double-blind and placebo controlled trial[32]. The safety and tolerance of Probio-Tec AB-25 and the placebo were good. Gastrointestinal symptoms were reported equally in both treatment groups and a relationship between Probio-Tec 25 and gastrointestinal side effects could not be established. At weeks 4 and 28, Bb-12 or La-5 were detected in 11 patients receiving probiotics. Five patients in the probiotic group (25%) and one patient in the placebo group (8%) maintained remission after 1 year of treatment. In the probiotic group, the median time to relapse was 125.5 days, versus 104 days in the placebo group. The use of BIFICO (oral capsules of live enterococci, bifidobacteria, and lactobacilli) in combination with sulphasalazine and glucocorticoid exerts some beneficial effects in preventing the relapse of UC (ref.[33]). The administration of BIFICO plus sulphasalazine and glucocorticoid to UC patients enlarged the number of bifidobacteria and lactobacilli and reduced the number of enterococci, bacteroides, and bifidobacteria present in the faeces compared with the control group. The most studied probiotic in clinical trials is Lactobacillus rhamnosus, which is present in the bowel of healthy individuals. Zocco et al. (ref.[34]) studied the efficacy of Lactobacillus rhamnosus GG (LGG) supplementation versus standard mesalazine for maintaining disease remission in UC patients. After 6 and 12 months of treatment, the percentage of patients maintaining clinical remission was, respectively, 91% and 85% for the LGG group (1.8 × 1010 viable bacteria/day), 87% and 80% for the mesalazine group (2400 mg/day), and 94% and 84% for the combined treatment (LGG plus mesalazine). The oral administration of Lacteol (Lacteol Fort, Rameda, Egypt), a probiotic preparation that contains 1 × 1010 CFU of Lactobacillus delbrueckii and Lactobacillus fermentum, together with 2400 mg/day of sulfasalazine, during 8 weeks, to UC patients with chronic diarrhea, inhibited the extent of inflammation, prevented mucosal injury, and alleviated colitis[35]. In children with distal active UC, rectal administration of Lactobacillus reuteri ATCC 55730 (as an enema solution containing 1 × 1010 CFU) for 8 weeks in addition to standard oral mesalazine, resulted in a significant decrease in the MDAI compared with the children that received the corresponding placebo. In addition, all of the children on Lactobacillus reuteri had a clinical response, whereas only 53% of the children on the placebo responded. Clinical remission was achieved in 31% of the Lactobacillus reuteri group and in no children of the placebo group[36]. D'Incà et al. (ref.[37]) evaluated the effect of an 8-week oral and/or rectal administration of Lactobacillus casei DG on colonic-associated microbiota, mucosal cytokine balance, and TLR expression in patients with mild left-sided UC. The patients were divided into three groups: the first group received oral 5-ASA alone, the second group received oral 5-ASA plus oral Lactobacillus casei DG (8 × 108 CFU), and the third group received oral 5-ASA and rectal Lactobacillus casei DG (8 × 108 CFU). A significant improvement of the histological scores was found in patients receiving the probiotic strain by the oral or rectal route of administration. Nevertheless, oral supplementation with Lactobacillus casei DG did not have a significant effect on the counts of Enterobacteriaceae or Lactobacillus. However, the occurrence of Lactobacillus and Enterobacteriaceae cultured from biopsy specimens was increased and decreased, respectively, in the group that took the probiotic rectally. Moreover, the rectal administration of Lactobacillus casei DG significantly reduced TLR-4 and IL-1β levels and significantly increased mucosal IL-10.

Probiotics act into the inflamed intestine, destroying "bad" bacterial flora and restoring previous micro-environment conditions. Probably, such an effect this prevents that abnormal reaction of the human immune system at

the base of intestinal autoimmune diseases. In this case, patients treated with probiotics showed an overall improvement of all studied parameters: patients showed a better clinical response, reduced significantly their stool frequency, maintained easily their haemoglobin values and exhibited a significant improvement of their gut mucosa condition. Likely, a random choice of probiotic strains should not be the right way to cure IBD definitely, but the present study demonstrates the efficacy of some "good" bacterial strains in assisting anti-inflammatory drug mechanism of action.

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