



Effect of probiotics on obesity-related markers per enterotype: a double-blind, placebo-controlled, randomized clinical trial

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Abstract

Background Prevention and improvement of disease symptoms are important issues, and probiotics are suggested as a good treatment for controlling the obesity. Human gut microbiota has different community structures. Because gut microbial composition is assumed to be linked to probiotic function, this study evaluated the efficacy of probiotics on obesity-related clinical markers according to gut microbial enterotype.

Methods Fifty subjects with body mass index over 25 kg/m² were randomly assigned to either the probiotic or placebo group. Each group received either unlabeled placebo or probiotic capsules for 12 weeks. Body weight, waist circumference, and body composition were measured every 3 weeks. Using computed tomography, total abdominal fat area and visceral fat area were measured. Blood and fecal samples were collected before and after the intervention for biochemical parameters and gut microbial compositions analysis.

Results Gut microbial compositions of all the subjects were classified into two enterotypes according to *Prevotella/Bacteroides* ratio. The fat percentage, blood glucose, and insulin significantly increased in the *Prevotella*-rich enterotype of the placebo group. The obesity-related markers, such as waist circumference, total fat area, visceral fat, and ratio of visceral to subcutaneous fat area, were significantly reduced in the probiotic group. The decrease of obesity-related markers was greater in the *Prevotella*-rich enterotype than in the *Bacteroides*-rich enterotype.

Conclusion Administration of probiotics improved obesity-related markers in obese people, and the efficacy of probiotics differed per gut microbial enterotype and greater responses were observed in the *Prevotella*-dominant enterotype.

Keywords Predictive preventive personalized medicine · Probiotics · Obesity · Clinical trial · Gut microbial enterotype

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Introduction

The prevalence of obesity has risen over the last few decades and has become a public health concern [1]. Prevention and improvement of obesity are important issues because obesity is not only a problem of its own, but it can be a risk factor for metabolic syndromes such as diabetes and cardiovascular disease [2, 3]. Recently, evidence that support obesity itself can increase mortality risk is increasing. And now it is more obvious that obesity itself can increase mortality risk. Study result of more than 10.1 million subjects from 239 large studies have shown that being overweight and obese is associated with higher risk of dying prematurely than normal weight [4]. Obesity also has a significant impact on the development of musculoskeletal diseases, such as osteoarthritis, rheumatoid arthritis, and fibromyalgia, by causing degenerative and inflammatory changes [5]. Fat accumulation in the liver, so-called hepatic steatosis, causes various hepatobiliary diseases, and

obesity is independently associated with liver fibrosis due to pathophysiological changes in immune system [6, 7]. Thus, obesity itself and immune response, such as chronic low-grade inflammation caused by obesity, are important factors causing various diseases. Obesity causes chronic low-grade inflammation because adipocytokines such as IL-1, IL-6, IL-8, IFN- γ , TNF- α and adipose tissue-secreted hormones such as leptin and resistin, induce the inflammation to become chronic [8]. Leptin promote Th1 responses which disrupt the Th1/Th2 immunity balance [9, 10]. Toll-like receptor 4 signaling pathway has been recognized as one of the main triggers in increasing the obesity-induced inflammatory response [11]. These obesity-induced inflammatory condition can lead to develop the cardiovascular diseases, type 2 diabetes mellitus, and certain types of cancer [12–14].

Various factors are involved in the pathophysiology of obesity, such as energy balance, genetic factors, epigenetic factors, and gut microbiota [15–17]. Gut microbiota influences nutrient absorption, energy balance, and the immunologic system, which contribute to modulation of body homeostasis and metabolism [18–20]. Associations of obesity and gut microbial imbalance have been extensively studied in human and animal models [21–23]. Gut microbiome has a potential role in predicting and validating the personalized responses to diet and variability of human metabolism in precision nutrition research field [24, 25]. Recent study reported the combined application of inorganic prebiotics and probiotics demonstrating a great clinical potential to treat hypercholesterolemia in affected individuals [26]. Cholesterol metabolism is sensitive to microbiota profiles and the setup of appropriate probiotic, immunobiotic, and prebiotic is crucial for the personalized clinical set [26]. Modulation of the gut microbiota and probiotic therapy can be important strategies in predictive, preventive, and personalized medicine to prevent the development of obesity and metabolic syndrome.

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” and provide health benefits such as inhibiting colonization of pathogens, modulating cholesterol level and treatment of diseases (e.g., obesity, diabetes, non-alcoholic fatty liver disease) [27–30]. *Lactobacillus* and *Bifidobacterium* are genus containing representative probiotics. Lactic acid bacteria including *Lactobacillus* have been reported to have functions related with inhibition of pathogens colonization, cholesterol metabolism, liver function, and fat accumulation [31–35], and *Bifidobacterium* have been reported to promote human health by decreasing weight and cholesterol, restoring liver morphology, modulating the gut microbiota, utilizing the human milk oligosaccharides, and enhancing the immune system [31, 36–38].

Human gut microbiota has different community structures depending on various factors such as diet, age, and geography [39, 40]. It has been suggested that human gut microbial

communities are mainly grouped into so-called enterotypes, depending on the dominant genus, such as *Prevotella*-dominant enterotype and *Bacteroides*-dominant enterotype [41, 42]. Enterotypes dominated by *Ruminococcus* or *Enterobacteriaceae* also occur, depending on the country or diet, but these are rare or ambiguous [43, 44]. There are some studies on the association of enterotype with cardiometabolic risk factors [45, 46] and the differential response to obesity-related markers, such as weight and fat mass [47, 48]. Therefore, selection of adequate probiotics for each person per their enterotype can increase their health promotion or disease preventing effects.

The aim of this study was to evaluate whether multi-strain probiotics improved obesity-related markers of subjects and to investigate the different responses per human gut microbial enterotype. The results of this study could be used for predictive diagnostics based on each individual's enterotype information and establishing a targeted prevention strategy by predicting the progression of obesity for each enterotype. It is also expected to be utilized for personalized medical services according to enterotypes including medical care and medical foods.

Materials and methods

Study design

Fifty healthy obese men and women participated in a 12-week double-blind, placebo-controlled, randomized clinical trial. This study investigated the effect of probiotic supplementation for body weight control. Subject recruitment and intervention proceeded from July 23, 2016 to February 14, 2017. Subjects received either probiotics or placebo for 12 weeks. Every 3 weeks, subjects visited for anthropometrical measurements and received probiotic or placebo capsules. At every visit, subjects were thoroughly surveyed for possible adverse events.

Subject eligibility criteria

Regarding inclusion and exclusion criteria, fifty subjects between 20 and 60 of age were recruited through advertisements in posters and local newspapers. In this trial, obesity was defined as those with a body mass index (BMI) over 25 kg/m², according to the Asia-Pacific Obesity guidelines [49]. Subjects who agreed to follow the study protocol for 12 weeks and voluntarily signed informed consent were included in this study.

Subjects with the following conditions were excluded from this study: (1) weight change more than 10% during the past 6 months; (2) weight-influencing diseases such as hypothyroidism and Cushing syndrome; (3) chronic diseases such as

heart diseases, cancer, pulmonary diseases, cholelithiasis, narrow angle glaucoma, and psychogenic diseases; (4) serious functional failure of liver and kidney (aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations more than 2.5 times the normal values, creatinine above 2.0 mg/dl); (5) undergone surgery for weight loss, such as bypass surgery; (6) history of stroke or ischemic heart diseases; (7) history of eating disorders such as anorexia nervosa or sitomania; (8) taking drugs such as hormonal drugs, selective serotonin reuptake inhibitors, central nervous system stimulants, or herbal medicine within 4 weeks; (9) history of taking anorectic agents, laxatives, steroids, thyroid hormones, amphetamine, or drugs that may affect body weight within 3 months; (10) history of taking probiotics for antibiotics within a month; (11) smokers or non-smokers who recently quit smoking within 3 months; (12) pregnant women, or who are planning to have a baby; or (13) participated in another clinical trial during the last 4 weeks.

Treatments

Following more than 10 days of run-in period, subjects took probiotic capsules or placebo capsules for 12 weeks. Subjects consumed two capsules per day after breakfast. The probiotic capsules, GP2 (Cell Biotech Co. Ltd., Gimpo, Republic of Korea), contained a formulation consisting of *B. breve* CBT BR3 isolated from Korean infant feces (15 billion viable cells/2 capsules), *L. plantarum* CBT LP3 isolated from Korean fermented vegetable product kimchi (15 billion viable cells/2 capsules), fructo-oligosaccharide, and magnesium stearate. Placebo capsules contained only fructo-oligosaccharide and magnesium stearate, but were identical in appearance.

Randomization and blinding strategies

Subjects were randomly allocated to either probiotic group or placebo group by a random code issued by an independent statistician. Random code was generated using Random Allocation Software (M. Saghaei, Iran) and sent to the manufacturer for packing. To maintain blindness on both the assessors and the subjects, probiotic and placebo capsules were packed and labeled with a provided random number before delivery to the hospital. Probiotic and placebo capsules were manufactured by Cell Biotech Co. (Gimpo, Republic of Korea), following the Korea Good Manufacturing Practice (KGMP). Placebo capsules had the same size and color as the probiotic capsules, so subjects could not differentiate the capsules. The pharmacist supplied the drug sequentially according to the random code. The blinding maintained until all the subjects had completed the study to minimize the risk of bias.

Energy intake measurements

Dietary guidelines suggested that subjects maintain a caloric restriction diet throughout the study, limiting caloric intake to 20–25 kcal per subject weight (kg). Although the subjects were not forced to maintain a caloric restriction, diets representing daily life were examined to determine whether there have been any changes during the study. Dietary intake was assessed before and after the trial, using the 24-h dietary recall method. Total energy consumption and nutrient intake were calculated using CAN-Pro 4.0 nutrient analysis software (The Korean Nutrition Society, Republic of Korea). Subjects were instructed to maintain their usual exercise program throughout the intervention. In order to rule out weight loss by exercise, people who did not exercise normally instructed to maintain their lifestyle.

Anthropometric parameters and body compositions measurements

Anthropometrical measurements were performed at every visit (Table 1). Body weight and height were measured with an automatic scale (G-tech, Uijeongbu, Republic of Korea). BMI was calculated by weight in kilograms divided by height in meters squared. Body composition measurements were determined using InBody 3.0 (Biospace, Seoul, Republic of Korea), a bioelectrical impedance analysis device to measure fat percentage, fat mass, and lean body mass. Blood pressure and pulse rate were measured using an automatic digital sphygmomanometer. To reduce errors, waist circumference (WC) and hip circumference (HC) were measured three times in a row by the same person and mean value was used for statistical analysis. WC was measured according to WHO instructions [50]. HC was measured at the widest portion of the buttocks.

Table 1 Clinical trial schedule

Schedules	Screening	Intervention				
		0	3	6	9	12
Time points (weeks)	> 10 days	0	3	6	9	12
Anthropometry	○	○	○	○	○	○
P/E	○	○	○	○	○	○
BIA	○	○		○		○
Fecal collection		○				○
Blood collection		○				○
Abdominal CT		○				○
24-h dietary recall		○				○

P/E physical examination, BIA bioelectrical impedance analysis, CT computed tomography

Abdominal fat measurements

Abdominal adiposity and visceral fat adiposity were measured before and after the study by computed tomography. Total abdominal fat area and visceral fat area were measured in a single transverse image taken at the level of L4–5 intervertebral space [51]. Subcutaneous fat area was obtained by subtracting visceral fat area from total abdominal fat area.

Blood chemistry

After overnight fasting, blood samples were collected before and after the intervention. Fasting glucose, insulin, triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), C-reactive protein, AST, and ALT were measured using Cobas 8000 modular analyzer (Roche, Branford, CT, USA). Low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald equation [52]: $LDL-C = (TC - HDL-C) - (TG/5)$.

Sequence-based microbiota analysis

Fecal samples were collected before and after the intervention and immediately stored at $-80\text{ }^{\circ}\text{C}$. Metagenomic DNA was isolated from the fecal samples using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), per manufacturer's instructions with an additional bead-beating step [53]. The V3–V4 regions of the 16S rRNA genes were amplified using the Illumina universal primers (341F and 806R). Amplified samples were sequenced on Illumina MiSeq platform using 2×300 bp MiSeq Reagent Kit V3 (Illumina, San Diego, CA, USA). Raw sequence reads were quality-filtered using Trimmomatic software [54], and read pairs passing the quality filter were merged using PEAR software [55]. Chimeras were identified using USEARCH software and removed from the sequence data [56]. Quality-controlled reads were processed to alpha and beta diversity analysis using QIIME2 pipelines [57]. All raw sequencing data described in this study are available from the DNA Data Bank of Japan with the accession number DRA008223.

Serum metabolite profiling

We analyzed serum metabolites using ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (Synapt G2Si, Waters, USA). A total of 200 μL of isopropanol was added to 50 μL of serum samples. Supernatants were obtained and diluted in half with deionized water; then, they were injected into an ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometer (UPLC-Q/TOF-MS, Synapt G2Si, Waters, USA). Acquity UPLC BEH C18 column (2.1 mm \times 100 mm, 1.7 μm , Waters) was used for separation. Mobile phase A

contained 0.1% formic acid in acetonitrile:water (6:4) and 10 nM ammonium formate. Mobile phase B consisted of 0.1% formic acid in isopropanol:acetonitrile (9:1) and 10 mM ammonium formate. Samples were eluted in 40 to 53% B for 2 min, 2.1 min to 50% A, 12 min to 54% B, 12.1 min to 70% B, 18 min to 1% B, 18.1 min to 40% B, and equilibrated for additional 2 min with the flow rate of 0.4 ml/min. Column temperature was retained at $55\text{ }^{\circ}\text{C}$. Mass acquisition was performed on a Q/TOF-MS (Synapt HDMS system, Waters) operating in both positive (ESI+) and negative (ESI-) electrospray ionization mode with following conditions: cone voltage of 10 V; capillary voltage of 2.0 kV; desolvation temperature was $550\text{ }^{\circ}\text{C}$; source temperature was $110\text{ }^{\circ}\text{C}$; and desolvation gas flow was 900 L/h. The range of m/z was 60 to 1400, mass data were collected with a scan time of 0.25 s, and interscan time of 0.02 s for 12 min. Information from mass data, including retention time, ion intensity, and m/z , were extracted using Progenesis QI software (Waters). After alignment and normalization, the multivariate data matrix was exported into SIMCA-P ver. 14.1+ (Umetrics, Umea, Sweden) for multivariate statistical analysis. The ions contributing to the separation of probiotic and placebo groups among female subjects over age 45 were further investigated by searching the METLIN database (<http://metlin.scripps.edu/>) and human metabolomics database (<http://www.hmdb.ca/>).

Statistical analyses

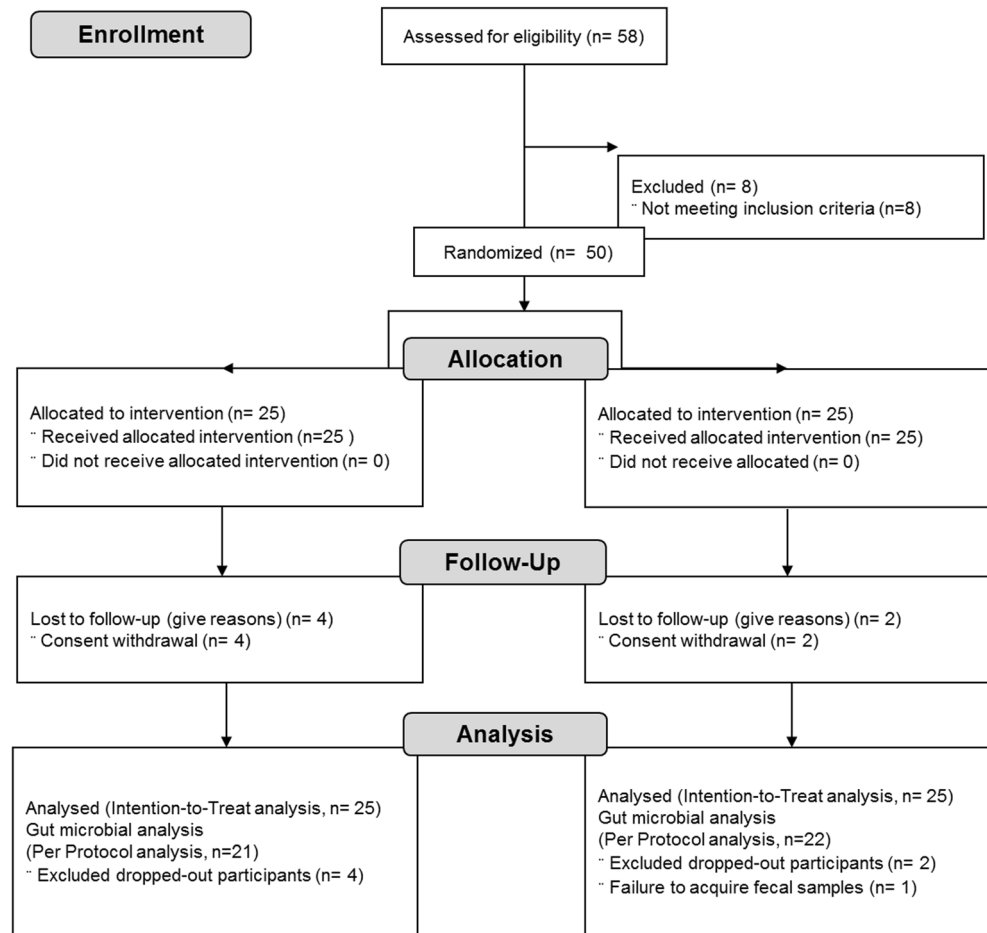
For statistical analyses of clinical parameters, intention-to-treat (ITT) analysis was used with the last observation carried forward method. Fisher's exact test was used for categorical data, such as gender. For continuous data, statistical analyses were done after verifying normality. Independent t test or Mann-Whitney test were used for the comparative analyses between two groups. We used paired t test or Wilcoxon signed rank test to compare pre-post results within groups. Statistical analyses were performed with SPSS statistical software package 18.0 (SPSS Inc., Chicago, IL, USA). A p value less than 0.05 was considered statistically significant.

Results

Study participants and adverse reactions

We screened and enrolled 50 subjects (Fig. 1), and they were randomly allocated to either probiotic group ($n = 25$) or placebo group ($n = 25$). There were no differences in baseline characteristics between the two groups (Table 2). During the study, four subjects from the probiotic group and two from the placebo group withdrew consent for personal reasons.

Fig. 1 Participant flowchart



Adverse events were reported from two patients in the probiotic group. One patient experienced dyspepsia and diarrhea, but recovered completely without any treatment. The other patient reported wrist sprain which is irrelevant to this study.

Dietary analysis

The investigators did not strictly encouraged participants to maintain caloric restriction nor give them specific guidelines for food, and our result is difficult to infer how eating habits have changed. But 24-h dietary recall analysis was investigated before and after the intervention to confirm whether subjects maintained a caloric restriction diet (Table 3). Results showed that there was no significant difference in energy intake between the two groups. Although energy intake did not change throughout the study, fat consumption significantly increased in both groups (mean difference of 2.35 g in the probiotic group and 3.77 g in the placebo group, $p < 0.0001$ for both groups). No significant difference in mineral intake was observed between two groups (Table 4). In both groups, calcium intake significantly

increased (13.65 mg in the probiotic group and 27.48 mg in the placebo group), while iron intake significantly decreased (0.21 mg in the probiotic group and 0.64 mg in the placebo group, $p < 0.0001$). There was no significant change in vitamin intake, except for vitamin C (Table 5), which increased significantly in the probiotic group (mean difference of 24.49 mg, $p = 0.027$).

Body weight and composition changes

The primary outcome of this study was measured by body weight and body composition changes after 12 weeks of intervention (Table 6). The probiotic group showed a significant decrease in waist circumferences (mean difference of 1.88 cm, $p = 0.049$), while the fat percentage of the placebo group significantly increased (mean difference of 1.14%, $p = 0.005$). Internal fat area and ratio of visceral to subcutaneous fat area (V/S ratio) significantly decreased in both groups. Internal fat area decreased by an average of 6.47 cm² in the probiotic group ($p < 0.001$) and 4.98 cm² in the placebo group ($p = 0.021$). The V/S ratio dropped by 0.022 in the

Table 2 Baseline characteristics

Variables	Probiotics (<i>n</i> = 25)	Placebo (<i>n</i> = 25)	<i>p</i> value†
Age (years)	42.92 ± 9.00	44.92 ± 9.34	.460
Height (cm)	163.03 ± 6.72	162.66 ± 5.80	.833‡
Gender (male/female)	5/20	3/22	.440§
Body weight (kg)	77.39 ± 13.42	75.67 ± 8.40	.705
BMI (kg/m ²)	29.02 ± 3.89	28.58 ± 2.74	.877
WC (cm)	98.52 ± 10.73	97.29 ± 5.47	.611‡
HC (cm)	105.66 ± 6.53	104.94 ± 4.75	.697
Fat mass (kg)	28.52 ± 7.00	28.26 ± 5.62	.889‡
Fat percentage (%)	36.81 ± 5.89	37.20 ± 5.07	.800‡
Systolic BP (mmHg)	133.36 ± 15.63	126.00 ± 13.48	.081‡
Diastolic BP (mmHg)	81.12 ± 8.96	77.32 ± 11.62	.202‡
Pulse rate (/min)	77.56 ± 11.46	77.16 ± 7.72	.885‡
Glucose (mg/dL)	97.36 ± 9.47	95.36 ± 8.66	.954
TG (mg/dL)	124.36 ± 59.50	112.24 ± 54.97	.388
TC (mg/dL)	198.80 ± 32.33	194.24 ± 36.31	.473
HDL-C (mg/dL)	56.92 ± 14.84	59.84 ± 14.58	.486‡
TC/HDL ratio	3.75 ± 1.23	3.46 ± 1.13	.390‡
LDL-C (mg/dL)	117.01 ± 31.53	111.95 ± 36.25	.691‡
AST (IU/L)	20.64 ± 10.24	21.80 ± 11.24	.816
ALT (IU/L)	25.16 ± 20.74	24.88 ± 18.49	.923
Insulin (μIU/mL)	10.96 ± 5.06	9.83 ± 7.80	.109
CRP (mg/dL)	0.152 ± 0.203	0.198 ± 0.475	.489

BMI body mass index, *WC* waist circumference, *HC* hip circumference, *BP* blood pressure, *TG* triglyceride, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *CRP* C-reactive protein

†*P* value by Mann-Whitney test

‡*P* value by independent *t* test

§ Fisher's exact test

**p* < 0.05

probiotic group (*p* < 0.001) and 0.015 in the placebo group (*p* = 0.013). The pulse rate of the probiotic group significantly decreased (mean difference of 3.4, *p* = 0.009).

Biochemical analyses

After 12 weeks of intervention, blood analyses indicated that there was no significant difference between two groups (Table 7). However, a few minor changes occurred. Fasting glucose concentration increased in the placebo group (mean difference of 3.88, *p* = 0.023). HDL-C concentration significantly increased in both the probiotic and placebo groups (mean differences of 2.72 and 5.28, respectively). Due to increased HDL-C concentration, total cholesterol/HDL-C ratio significantly increased in both groups. However, there were no significant differences between the two groups.

Gut microbial community analysis

Although our clinical results were based on ITT analysis, we performed a gut microbial analysis on pre- and post-intervention stool samples. Due to failure to acquire a fecal sample, one subject from the placebo group was excluded from this analysis.

Compositional changes in gut microbiota by probiotic or placebo supplementation were analyzed using 16S rRNA gene sequence analysis (Fig. 2). The Chao1 indices slightly decreased after administration of probiotic (*p* = 0.04) or placebo capsule (*p* = 0.0078). The Shannon and Simpson indices did not change significantly (Fig. 2a). Non-metric multidimensional scaling (NMDS) based on the Bray–Curtis distance matrices showed no obvious separation between the groups (Fig. 2b). There were no significant differences between groups or before–after comparison at phylum and family levels (Fig. 2c).

When clustering gut microbiota before intervention, it was divided into two enterotypes according to *Prevotella/Bacteroides* ratio (Fig. 3a). Enterotype 1 (Ent1) had a relatively higher proportion of *Prevotella* (average = 24.18%, SD = 15.70%) than *Bacteroides* (average = 10.33%, SD = 5.97%). In the other hand, Enterotype 2 (Ent2) had relatively lower *Prevotella* (average = 0.55%, SD = 1.57%) than *Bacteroides* (average = 32.43%, SD = 14.43%). The Chao1 richness estimator significantly decreased in Ent1 after administering probiotic (*p* = 0.044) or placebo capsule (*p* = 0.0035) (Fig. 3b). While NMDS based on the Bray–Curtis distance matrices showed no obvious separation between the groups, the change in the Bray–Curtis distance of Ent1 after administering probiotics was significantly higher than the other group (Fig. 3c). Firmicutes and Bacteroidetes, as well as other rare phyla, were not significantly different between the probiotic–placebo, before–after, or Ent1–Ent2 comparisons (Fig. 3d). However, the relative abundance of Prevotellaceae in the Ent1 of the probiotic group was diminished—from an average of 35.66% (SD = 15.29%) before intervention to 20.99% (SD = 17.73%) after—but it was not significant.

Differences in probiotic response per microbial enterotype

The differences in response per enterotype were identified by changes in waist circumferences and body fat composition (Table 8). Total fat area was significantly less in the Ent1 group with probiotics (mean difference of 19.46 cm², *p* = 0.040), whereas fat percentage was significantly greater in the Ent1 of the placebo group (mean difference of 1.27%, *p* = 0.015). Internal fat area and V/S ratio significantly decreased in both Ent1 and Ent2 of the probiotic group. However, comparing Ent1 and Ent2 within probiotics group showed that internal fat area reduced much more in the Ent1

Table 3 Dietary intake between the probiotic and placebo group, from the 24-h dietary recall analysis

Variables	Time	Probiotics group (n = 25)		Placebo group (n = 25)		Within‡ (probiotics)	Within‡ (placebo)	P value§
		Mean	SD	Mean	SD			
Energy intake (kcal)	Baseline	1195.73	453.31	1281.54	415.73			.49
	12 weeks	1195.60	412.72	1200.08	371.67	≥ .99	.46	.97
Protein (g)	Baseline	69.84	28.41	86.28	87.12			.37
	12 weeks	70.92	23.68	87.12	16.19	.72	.50	.56
Fat (g)	Baseline	30.26	19.00	35.37	18.96			.35
	12 weeks	32.61	16.23	39.14	16.36	< .001*	< .001*	.16
Carbohydrate (g)	Baseline	181.81	59.69	187.61	64.81			.74
	12 weeks	178.66	70.54	161.43	70.02	.82	.18	.39
Dietary fiber (g)	Baseline	10.74	5.04	11.55	4.33			.55
	12 weeks	11.25	4.26	10.67	4.37	.64	.44	.64
Cholesterol (mg)	Baseline	223.33	177.90	221.71	160.93			.97
	12 weeks	188.39	155.03	259.82	166.73	.28	.45	.12
Total fatty acid (g)	Baseline	15.68	12.57	19.10	10.75			.31
	12 weeks	18.63	12.51	23.98	18.05	.41	.16	.31

†P value by Wilcoxon signed rank test

‡P value by paired t test

§ P value by Mann-Whitney test or independent t test (energy intake, lipid, carbohydrate, and dietary fiber)

*p < 0.05

group, by an average of 12.50 cm² (p = 0.002), compared with the Ent2 group, by an average of 4.10 cm² (p = 0.029). The differential response according to enterotype was also identified by changes in biochemical markers (Table 9). TG

significantly decreased in Ent1 of the probiotic group (mean difference of 16.22 mg/dL, p = 0.022). TC/HDL significantly decreased in Ent1 of the probiotic group (mean difference of 7.92, p = 0.036). Glucose

Table 4 Mineral intake between probiotic and placebo groups, from the 24-h dietary recall analysis

Variables	Time	Probiotics group (n = 25)		Placebo group (n = 25)		Within‡ (probiotics)	Within‡ (placebo)	P value§
		Mean	SD	Mean	SD			
Calcium (mg)	Baseline	299.57	180.88	292.75	154.68			.95
	12 weeks	313.22	146.57	320.23	131.93	< .001†*	< .001*	.86
Phosphorus (mg)	Baseline	620.98	288.69	640.13	190.92			.78
	12 weeks	646.44	283.92	669.24	252.61	.52†	.98†	.65
Iron (mg)	Baseline	9.20	5.18	8.39	2.96			.85
	12 weeks	8.99	3.83	7.75	3.13	< .001†*	< .001*	.22
Sodium (mg)	Baseline	2416.48	1208.90	2386.26	622.60			.40
	12 weeks	2341.68	910.96	2634.20	1425.67	.82†	.46	.39
Potassium (mg)	Baseline	1412.86	687.28	1622.69	453.08			.21
	12 weeks	1524.12	653.86	1446.63	477.39	.46	.13	.63
Zinc (mg)	Baseline	9.04	15.96	5.94	1.98			.76
	12 weeks	5.64	2.09	5.36	1.56	.24†	.21	.59

†P value by Wilcoxon signed rank test

‡P value by paired t test

§ P value by Mann-Whitney test or independent t test (phosphorus and zinc)

*p < 0.05

Table 5 Vitamin intake between probiotic and placebo groups, from the 24-h dietary recall analysis

Variables	Time	Probiotics group (<i>n</i> = 25)		Placebo group (<i>n</i> = 25)		Within‡ (probiotics)	Within‡ (placebo)	<i>P</i> value§
		Mean	SD	Mean	SD			
Vitamin A (µg RE)	Baseline	601.77	804.56	721.12	843.61			.20
	12 weeks	445.42	273.80	547.87	632.72	.64†	.38†	.78
Vitamin B ₁ (mg)	Baseline	0.68	0.34	0.81	0.24			.12
	12 weeks	0.79	0.35	0.82	0.36	.19	.97	.80
Vitamin B ₂ (mg)	Baseline	0.74	0.53	0.76	0.37			.54
	12 weeks	0.74	0.41	0.74	0.34	.69†	.74†	.75
Vitamin B ₆ (mg)	Baseline	1.28	0.67	1.42	0.38			.40
	12 weeks	1.18	0.48	1.19	0.47	.61	.06	.93
Niacin (mg)	Baseline	10.78	6.15	10.92	3.84			.49
	12 weeks	10.43	4.56	10.80	3.96	.78	.74†	.76
Vitamin C (mg)	Baseline	36.76	21.82	42.72	19.54			.11
	12 weeks	51.05	25.72	41.06	17.99	.03†*	.77†	.12
Folate (µg)	Baseline	145.22	112.55	146.17	71.50			.35
	12 weeks	136.19	63.26	133.46	62.19	.39†	.25†	.88
Vitamin E (mg)	Baseline	7.32	5.06	9.69	5.34			.07
	12 weeks	8.16	4.39	9.98	5.71	.41†	.83†	.37

†*P* value by Wilcoxon signed rank test‡*P* value by paired *t* test§ *P* value by Mann-Whitney test or independent *t* test (vitamin B₁ and B₆)**p* < 0.05

significantly increased in Ent1 of the placebo group (mean difference of 5.37 mg/dL, *p* = 0.010). Finally, insulin significantly increased in Ent1 of the placebo group (mean difference of 2.47 µIU/mL, *p* = 0.0039).

Table 6 Anthropometric parameters, body composition and blood pressure after consumption of probiotic or placebo capsules

Variables	Probiotics group (<i>n</i> = 25)			Placebo group (<i>n</i> = 25)			<i>P</i> value§
	Initial	Final	Within‡	Initial	Final	Within‡	
Body weight (kg)	77.39 ± 13.42	76.89 ± 13.26	.223	75.67 ± 8.40	75.22 ± 8.58	.265	.600
BMI (kg/m ²)	29.02 ± 3.89	28.81 ± 3.71	.346†	28.58 ± 2.74	28.41 ± 2.79	.258	.668
WC (cm)	98.52 ± 10.73	96.64 ± 9.63	.049*	97.29 ± 5.47	96.38 ± 6.39	.173	.911
HC (cm)	105.66 ± 6.53	105.16 ± 6.35	.453†	104.94 ± 4.75	104.30 ± 4.88	.589†	.594
Fat mass (kg)	28.52 ± 7.00	28.28 ± 7.45	.695	28.26 ± 5.62	28.82 ± 5.69	.087	.776
Fat percentage (%)	36.81 ± 5.89	36.65 ± 6.36	.322†	37.20 ± 5.07	38.34 ± 5.12	.005*	.306
Total fat area (cm ²)	215.21 ± 62.06	208.25 ± 58.11	.114†	212.62 ± 48.11	208.82 ± 40.92	.300	.968
Visceral fat area (cm ²)	67.02 ± 22.48	60.55 ± 21.29	< .001*	71.57 ± 29.45	66.59 ± 24.80	.021*	.360
V/S ratio	0.318 ± 0.099	0.296 ± 0.095	< .001*	0.329 ± 0.096	0.314 ± 0.088	.013*	.485
Systolic BP (mmHg)	133.36 ± 15.63	130.96 ± 14.93	.432†	126.00 ± 13.48	125.88 ± 9.41	.964	.157
Diastolic BP (mmHg)	81.12 ± 8.96	81.52 ± 12.64	.822	77.32 ± 11.62	79.40 ± 9.40	.605†	.504
Pulse rate (/min)	77.56 ± 11.46	74.16 ± 10.01	.009*	77.16 ± 7.72	75.16 ± 9.06	.203	.713

BMI body mass index, *WC* waist circumference, *HC* hip circumference, *V/S ratio* ratio of visceral to subcutaneous fat area, *BP* blood pressure

†*P* value by Wilcoxon signed rank test‡*P* value by paired *t* test§ *P* value by Mann-Whitney test (body weight and BMI) or independent *t* test**p* < 0.05

Table 7 Biochemical analysis after 12 weeks of probiotic or placebo intervention

Variables	Probiotics group (n = 25)			Placebo group (n = 25)			p value [§]
	Initial	Final	Within‡	Initial	Final	Within‡	
Glucose (mg/dL)	97.36 ± 9.47	98.16 ± 11.66	.585	95.36 ± 8.66	99.24 ± 7.77	.023†	.217
TG (mg/dL)	124.36 ± 59.50	114.00 ± 62.50	.112	112.24 ± 54.97	102.88 ± 48.28	.361†	.509
TC (mg/dL)	198.80 ± 32.33	195.48 ± 37.36	.438	194.24 ± 36.31	200.04 ± 30.77	.183	.640
HDL-C (mg/dL)	56.92 ± 14.84	59.64 ± 15.91	.046†*	59.84 ± 14.58	65.12 ± 13.07	.003*	.190
LDL-C (mg/dL)	117.01 ± 31.53	113.04 ± 35.35	.297	111.95 ± 36.25	114.34 ± 29.87	.497	.889
TC/HDL ratio	3.75 ± 1.23	18.64 ± 9.72	< .0001*	3.46 ± 1.13	21.72 ± 10.45	< .0001*	.285
AST (IU/L)	20.64 ± 10.24	21.12 ± 9.39	.569	21.80 ± 11.24	20.68 ± 9.84	.295†	.726
ALT (IU/L)	25.16 ± 20.74	26.12 ± 21.43	.502	24.88 ± 18.49	23.32 ± 17.04	.243	.861
Insulin (μIU/mL)	10.96 ± 5.06	10.25 ± 4.59	.380	9.83 ± 7.80	10.21 ± 6.91	.743	.509
CRP (mg/dL)	0.152 ± 0.203	0.135 ± 0.155	.896†	0.198 ± 0.475	7.852 ± 26.681	.625†	.521

TG triglyceride, TC total cholesterol, HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, AST aspartate amino-transferase, ALT alanine aminotransferase, CRP C-reactive protein

*p < 0.05

Regardless of the allocated group, baseline clinical parameters were compared to determine if there were any biomarkers to distinguish between Ent1 and Ent2. There were statistically significant differences between two groups in baseline glucose and insulin level. However, the differences were not clinically noticeable.

Changes in plasma metabolites

Multivariate statistical analysis from the metabolic profiling was performed with plasma samples obtained from the subjects before and after the study. The principal component analysis of plasma spectral data did not show clear clustering. Therefore,

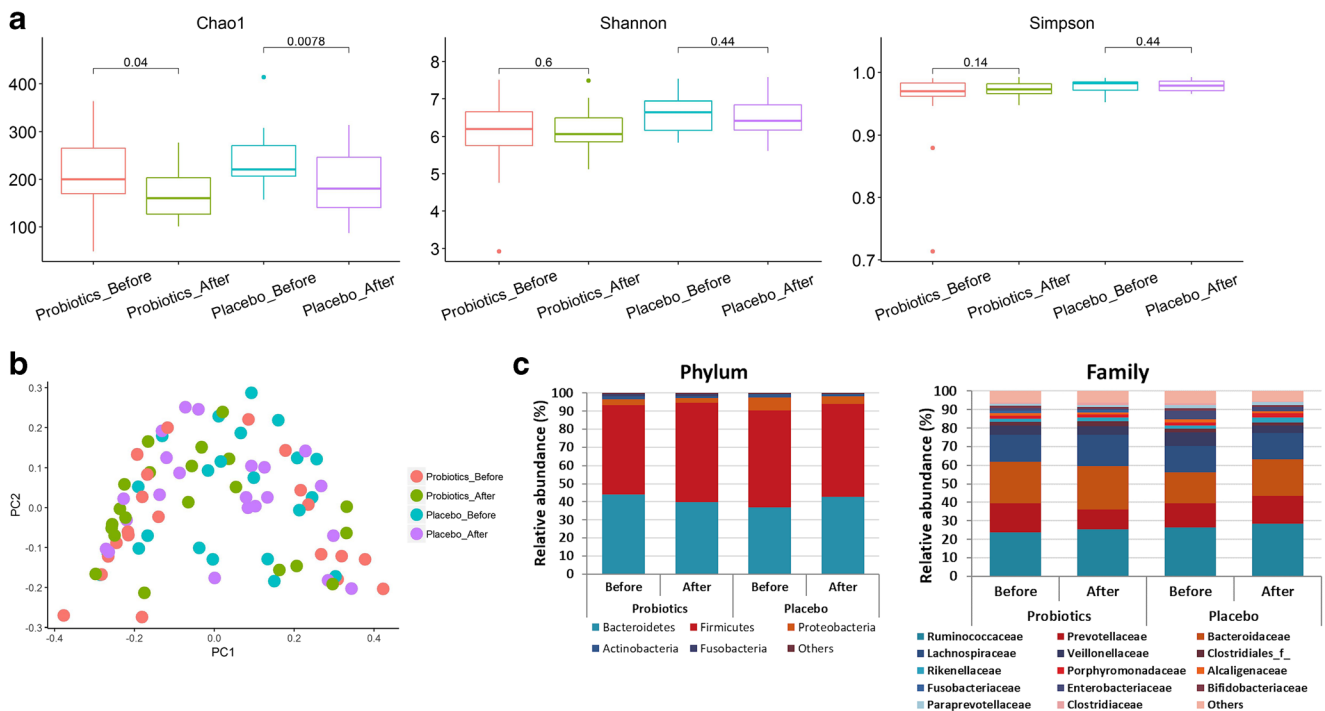


Fig. 2 Diversity analysis between groups and their changes during intervention. **a** Alpha diversity (Chao1, Shannon, and Simpson metrics). Lines in box represent median alpha diversities. The box ranges from the lower to the upper quartiles. Statistical significance was

determined by Wilcoxon paired *t* test. **b** Non-metric multidimensional scaling (NMDS) beta diversity plots using Bray–Curtis distance matrices. **c** Relative abundance of the top 5 most abundant phyla and the top 14 most abundant families in each group

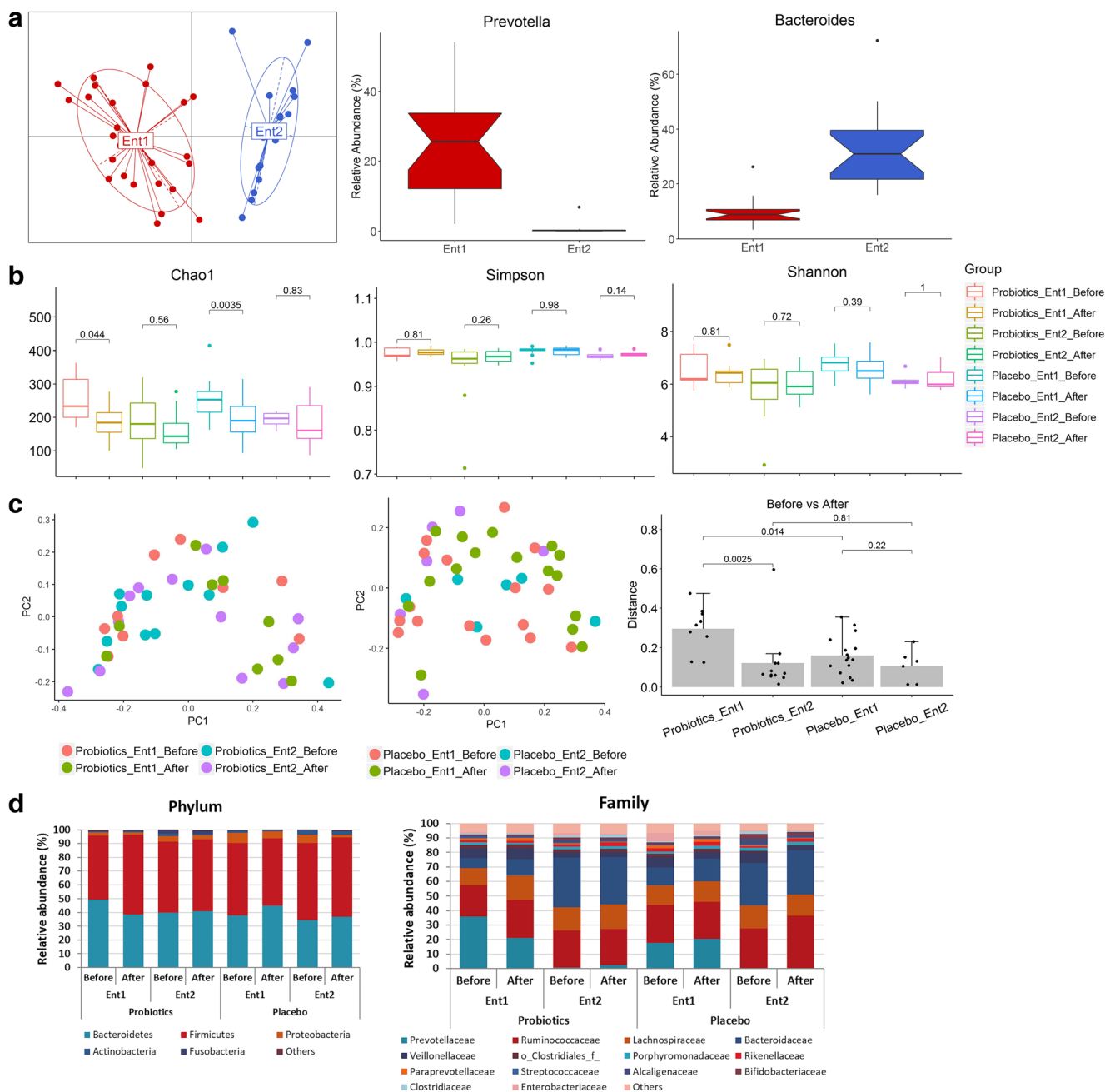


Fig. 3 Changes in microbial community according to enterotype. **a** Identified enterotypes and abundances of the main contributors for each enterotype. **b** Alpha diversity (Chao1, Shannon, and Simpson metrics). Lines in box represent median alpha diversities. The box ranges from the lower to the upper quartiles. Statistical significance was determined by

Wilcoxon paired *t* test. **c** Non-metric multidimensional scaling (NMS) beta diversities using Bray–Curtis distance matrices. Statistical significance was determined by Wilcoxon paired *t* test. **d** Relative abundance of the top 5 most abundant phyla and the top 14 most abundant families in each group

further analysis applied the orthogonal projections to latent structures discriminant analysis (OPLS-DA) model to distinctly demonstrate metabolite profiles (Fig. 4). The OPLS-DA score plots revealed that the probiotic group was not statistically distinguishable from the placebo group ($R^2X = 0.0803$, $R^2Y = 0.0409$ in the negative mode and $R^2X = 0.154$ and $R^2Y = 0.32$ in the positive mode). However, the probiotic and placebo groups after the study showed distinct clusters in the negative

mode of OPLS-DA score plots, but there were no distinct clusters between the groups before the study.

Discussion

In this double-blinded, placebo-controlled, randomized clinical trial, we found that a 12-week administration of

Table 8 Anthropometric parameters, body composition, and blood pressure after consumption of probiotic or placebo capsules per enterotype

Variables	Probiotics Ent1 (n = 9)			Probiotics Ent2 (n = 12)			Placebo Ent1 (n = 16)			Placebo Ent2 (n = 6)		
	Initial	Final	Within‡	Initial	Final	Within‡	Initial	Final	Within‡	Initial	Final	Within‡
Body weight (kg)	82.81 ± 17.16	81.88 ± 17.45	0.441	72.97 ± 8.11	73.15 ± 7.94	0.505	73.22 ± 8.78	73.47 ± 9.22	0.600	78.57 ± 6.93	77.93 ± 5.93	0.513
BMI (kg/m ²)	30.64 ± 4.95	30.29 ± 4.86	0.309	27.87 ± 3.01	27.93 ± 2.92	0.575	27.62 ± 2.27	27.73 ± 2.46	0.234	29.32 ± 3.36	29.10 ± 3.14	0.531
WC (cm)	100.72 ± 13.66	97.22 ± 11.12	0.151	96.67 ± 9.11	95.29 ± 8.89	0.195	96.19 ± 6.64	95.44 ± 6.92	0.330	98.50 ± 5.68	96.92 ± 5.97	0.271
HC (cm)	106.81 ± 8.49	106.44 ± 8.53	0.690	104.67 ± 6.14	104.25 ± 5.46	0.418	104.24 ± 3.56	103.69 ± 4.79	0.361	105.88 ± 6.18	104.83 ± 6.31	0.752
Fat mass (kg)	30.81 ± 7.69	30.36 ± 7.29	0.677	27.69 ± 6.96	26.91 ± 7.99	0.516	26.94 ± 4.75	27.81 ± 5.47	0.062	29.50 ± 7.07	29.72 ± 6.73	0.778
Fat percentage (%)	37.30 ± 5.30	37.12 ± 3.93	0.868	37.82 ± 7.21	36.52 ± 8.68	0.442	36.74 ± 4.49	38.01 ± 4.99	0.015‡	37.30 ± 6.88	37.88 ± 6.57	0.438
Total fat area (cm ²)	221.40 ± 67.44	201.94 ± 60.02	0.040‡	205.96 ± 61.45	206.05 ± 58.52	0.910	204.91 ± 49.33	204.14 ± 39.55	0.864	221.72 ± 54.19	212.03 ± 54.73	0.310
Visceral fat area (cm ²)	75.24 ± 25.99	62.74 ± 24.48	0.002‡	61.51 ± 21.40	57.41 ± 21.63	0.029‡	64.67 ± 30.82	61.14 ± 25.44	0.323	83.62 ± 20.50	75.12 ± 21.53	0.119
V/S ratio	0.34 ± 0.06	0.31 ± 0.06	0.049†	0.32 ± 0.13	0.29 ± 0.13	0.011†	0.31 ± 0.09	0.29 ± 0.08	0.054	0.38 ± 0.10	0.36 ± 0.08	0.104
Systolic BP (mmHg)	136.67 ± 16.04	133.44 ± 22.79	0.494	135.17 ± 14.98	131.33 ± 5.84	0.423	124.56 ± 14.67	123.62 ± 10.68	0.788	124.33 ± 12.36	129.00 ± 3.03	0.387
Diastolic BP (mmHg)	80.33 ± 13.08	82.44 ± 19.07	0.513	83.83 ± 8.64	81.17 ± 6.81	0.311	75.62 ± 9.73	77.44 ± 10.61	0.521	77.50 ± 10.17	82.33 ± 4.68	0.163
Pulse rate (/min)	70.67 ± 7.45	71.44 ± 11.76	0.751	78.00 ± 11.01	76.17 ± 10.14	0.325	73.00 ± 10.15	72.88 ± 10.56	0.963	79.83 ± 10.67	79.83 ± 3.19	1.000

BMI body mass index, WC waist circumference, HC hip circumference, V/S ratio ratio of visceral to subcutaneous fat area, BP blood pressure

†P value by Wilcoxon signed rank test

‡P value by paired t test

§ P value by Mann-Whitney test or independent t test

*p < 0.05

Table 9 Anthropometric parameters, body composition, and blood pressure after consumption of probiotic or placebo capsules per enterotype

Variables	Probiotics Ent1 (n = 9)			Probiotics Ent2 (n = 12)			Placebo Ent1 (n = 16)			Placebo Ent2 (n = 6)		
	Initial	Final	Within‡	Initial	Final	Within‡	Initial	Final	Within‡	Initial	Final	Within‡
Glucose (mg/dL)	95.89 ± 9.14	96.56 ± 9.75	0.787	100.42 ± 10.35	101.58 ± 13.91	0.953	92.44 ± 9.45	97.81 ± 8.60	0.010*	99.67 ± 3.01	100.67 ± 5.50	0.651
TG (mg/dL)	119.89 ± 49.90	103.67 ± 47.86	0.022*	137.50 ± 72.00	128.08 ± 78.81	0.530	95.38 ± 41.49	91.31 ± 43.21	0.628	131.50 ± 71.98	121.00 ± 53.47	0.421
TC (mg/dL)	202.78 ± 46.09	194.33 ± 51.98	0.439	197.92 ± 25.91	197.33 ± 32.19	0.896	194.62 ± 39.61	201.12 ± 33.45	0.214	183.67 ± 35.42	187.33 ± 27.26	0.779
HDL-C (mg/dL)	52.22 ± 14.25	54.67 ± 12.80	0.081	57.92 ± 16.05	61.75 ± 19.08	0.195	64.44 ± 12.50	69.62 ± 12.92	0.028*	49.83 ± 13.17	55.00 ± 6.69	0.009*
LDL-C (mg/dL)	4.17 ± 1.46	3.71 ± 1.23	0.453	3.67 ± 1.17	3.52 ± 1.31	0.443	3.16 ± 1.00	3.00 ± 0.87	0.582	3.82 ± 0.81	3.46 ± 0.72	0.958
TC/HDL ratio	126.58 ± 42.55	118.93 ± 46.43	0.036*	112.50 ± 26.56	109.97 ± 32.98	0.291	111.11 ± 40.45	113.24 ± 32.28	0.103	107.53 ± 33.77	108.13 ± 25.06	0.440
AST (IU/L)	19.11 ± 5.93	19.56 ± 7.76	0.794	22.92 ± 13.69	23.58 ± 11.42	0.532	22.69 ± 12.95	20.75 ± 10.70	0.170	22.50 ± 8.12	21.83 ± 10.36	0.763
ALT (IU/L)	24.78 ± 17.85	26.56 ± 25.00	0.513	27.58 ± 25.48	28.25 ± 22.06	0.665	25.81 ± 21.51	23.75 ± 19.04	0.333	26.67 ± 13.75	24.50 ± 16.63	0.408
Insulin (μU/mL)	11.60 ± 5.66	11.32 ± 4.89	0.881	10.99 ± 5.45	9.73 ± 5.05	0.238	8.00 ± 7.25	10.47 ± 8.15	0.039†	15.31 ± 9.15	10.77 ± 4.79	0.143
CRP (mg/dL)	0.06 ± 0.04	0.10 ± 0.11	0.407	0.23 ± 0.27	0.17 ± 0.20	0.625	0.24 ± 0.59	0.14 ± 0.20	0.865	0.12 ± 0.14	32.26 ± 49.76	0.590

TG triglyceride, TC total cholesterol, HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, AST aspartate aminotransferase, ALT alanine aminotransferase, CRP C-reactive protein

†P value by Wilcoxon signed rank test

‡P value by paired t test

§ P value by Mann-Whitney test or independent t test

*P < 0.05

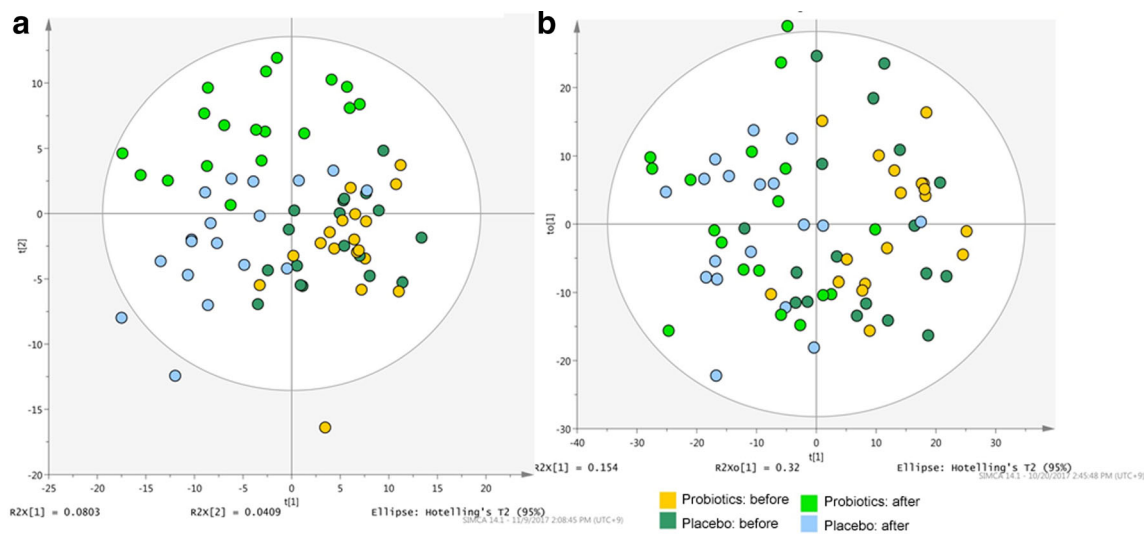


Fig. 4 Orthogonal projections to latent structures discriminant analysis (OPLS-DA) score plots based on serum spectral samples, obtained before and after taking either probiotic or placebo capsules for 12 weeks. **a** Operated in negative ion mode. **b** Operated in positive ion mode

a multispecies probiotic significantly changed obesity-related markers, such as waist circumference, total fat area, visceral fat, V/S ratio, and biochemical markers, such as TG, TC, HDL, glucose, and insulin. These changes were more remarkable in the *Prevotella*-rich enterotype than in the *Bacteroides*-rich enterotype.

Obesity-related markers in response to probiotics treatment

Although the effect on weight loss was not significant, the results showed an improvement in other factors related to obesity, such as WC and total fat area. Our result is notable, because among many obesity-related parameters suggested to explain risk factors of metabolic diseases, WC is associated with an increased risk for metabolic diseases when the BMI is between 25 and 34.9 [58, 59]. A similar result was observed in a study providing *Lactobacillus gasseri* BNR17 supplementation for 12 weeks—significant reductions in WC and HC in the probiotic group—while a slight increase in fat percentage was observed in the placebo group [60].

Considerable evidence has shown that amount of visceral fat is a key etiological factor of cardiovascular diseases [61, 62]. Focusing on fat distribution, V/S ratio has emerged as a potential risk factor [63, 64]. In the current study, prominent reductions in visceral fat area and V/S ratio were found in both the probiotic and placebo groups. The mechanisms involved in reduction of visceral fat are not clear, but a few studies have reported the possible mechanisms. There was a human study that demonstrated a cholesterol- and glucose-lowering effect of *L. plantarum* [65]. The result was accompanied by a significant reduction in interleukin 6. Another study reported that *Lactobacillus plantarum* strain No.14 had a beneficial effect on obese mice by reducing adipocyte size [66]. This may explain our study

result that showed reduction in WC was accompanied by a significant decrease in visceral fat. A similar study using a 12-week administration of *Bifidobacterium animalis* subsp. *lactis* GCL2505 for obese subjects found a significant decrease in abdominal visceral fat, which was accompanied by an increase in the total number of *Bifidobacterium* in fecal samples [67]. Another study—*L. gasseri* SBT2055 treatment for 12 weeks—showed beneficial effects on visceral fat area and WC, while the placebo group showed increasing body fat percentage and body fat mass [68].

Results from the 24-h dietary recall showed that there was no statistical difference between the two groups in energy intake. Interestingly, the intake of fat increased in both groups after the intervention. Despite the increased consumption of fat, obesity-related markers improved in the probiotic group, while the placebo group showed an increase in body fat percentage. This result is probably due to GP2, which presents a probiotic effect on obesity. For some reason, both groups showed significant decrease in iron intake and increase in calcium intake. Although our data from the 24-h dietary recall does not reflect habitual changes in diet, the changes of mineral intake are worthy of notice. Probiotics is known to have beneficial effect on host by increasing in the absorption of micronutrients such as calcium, iron, and magnesium [69]. An in vivo study found that a low-iron diet triggers iron-dependent microbiome, such as *Lactobacillus* species to absorb iron from the food and increase iron-storage protein ferritin [70]. In human studies, it is known that commensal bacteria do not need iron from the intestinal lumen, and rather excessive intake of iron leads to pathogenic intestinal flora. However, iron deficiency can cause a decrease in bacteria such as *Bacteroides* and *Roseburia* and an increase in *Lactobacillus* and *Enterobacteriaceae* [71]. In this context, low intake of iron in both groups might have affected bacterial

homeostasis and *Lactobacillus* supplementation might have helped the modulation in probiotics group but not in the placebo group which might have consequently affected overall clinical conditions. However, our data from the dietary recall is not directly related to amount of iron absorbed from the food. Future study to evaluate whether probiotic intake affects mineral absorption is needed.

***Bifidobacterium breve* and *Lactobacillus plantarum* strains as a probiotics**

The GP2, which we used in this study, is a multiple strain probiotic composed of *Bifidobacterium breve* and *Lactobacillus plantarum*. *Bifidobacterium* is capable of producing conjugated linoleic acids that have anti-obesity, anti-diabetic, anti-carcinogenic, and immune regulation properties [72–74]. Particularly, *Bifidobacterium breve* have effect on metabolic syndrome-suppressing and anti-obesity [75, 76]. *Lactobacillus* is classified as a lactic acid producing bacteria that play a major role in modulating gut ecology [77, 78]. Particularly, *L. plantarum* showed effect on treatment of vaginal disorders and reducing the body weight and cholesterol level [79–82]. Many experimental studies have clearly shown the anti-obesity effects of probiotics belonging to *Lactobacillus* and *Bifidobacterium* strains [83, 84].

Various probiotic characteristics such as gastric and bile acids tolerance and epithelial cell adhesion ability of currently used *Lactobacillus plantarum* CBT LP3 were previously reported [85]. Probiotic functions of *L. plantarum* CBT LP3 related to energy metabolism in obesity and reduction of atopic dermatitis symptoms were also previously reported [86, 87]. Currently used *Bifidobacterium breve* CBT BR3 strain was isolated from the feces of a healthy infant and several kinds of genetic factors associated with health promotion of the human host such as oligosaccharide degradation and vitamin biosynthesis were identified by complete genome sequence analysis of this strain [88]. Health promoting probiotic functions of *B. breve* CBT BR3 such as antimicrobial, anti-inflammatory, and anti-obesity activities also have been reported [87, 89–91]. For the development of anti-obesitic probiotics, we evaluated anti-obesity effect of various probiotic strains including *L. plantarum* CBT LP3 and *B. breve* CBT BR3 onto cell and animal model [92]. *L. plantarum* CBT LP3 and *B. breve* CBT BR3 showed higher efficacy than other tested strains and treatment of complex probiotics by these two strains was most effective. For the clinical trial, each volunteers served two probiotic capsules formulated with *L. plantarum* CBT LP3 BR3 (1.5×10^9 viable cells/2 capsules) and *B. breve* CBT BR3 (1.5×10^9 viable cells/2 capsules), and this daily dosage of *Lactobacillus* and *Bifidobacterium* spp. is accepted as probiotics when delivered in food at a level of 1×10^9 colony-forming units (CFU) per serving [93].

GP2 and placebo capsule contain fructo-oligosaccharide (FOS) and magnesium stearate. Magnesium stearate is

commonly used as an inactive ingredient in the production of pharmaceutical tablets, capsules, and powders [94]. Prebiotics are defined as “a substrate that is selectively utilized by the host microorganisms conferring a health benefit” by the dedicated International Scientific Association for Probiotics and Prebiotics (ISAPP) expert group [95]. Fructans (FOS and inulin) and galactans (galactooligosaccharides or GOS) have dominated the prebiotic category and recently, nanoceria can be a new prebiotics to reduce cholesterol levels and restore gut microbiota in obese mice when composed with probiotics [26]. Whereas probiotics use live microorganisms, prebiotics are non-viable substrates that serve as nutrients for administered probiotic strains and indigenous microorganisms [95, 96]. In addition, prebiotics can be used together with specified probiotic treatments by synergetic effect to treat obesity and other diseases in view of predictive, preventive, and personalized concepts [26]. Prediction and optimization of the pre- and probiotic composition from individualized patient profiles enable the personalized treatment.

Immunity systems: relevant pathways

It has been well established that the immune system plays an important role in the pathogenesis of obesity [97]. Disrupted gut barrier function in obesity increases bacterial translocation such as LPS and LPS-binding protein, which stimulate the inflammation in adipose tissue [98, 99]. T cells are involved primarily in the cell-mediated immune response and T helper (Th) cells are key regulators [100]. Th1 and Th2 cytokine imbalance are reported in various disease states such as autoimmune disorders, asthma, and adipose tissue inflammation [101–103]. Lean adipose tissue contains balanced level of Th2 and regulatory T (Treg) cells that maintain macrophage activity and inhibit proinflammatory cells through cytokines such as IL-10, whereas obese adipose tissue contains many infiltrated proinflammatory cells such as Th1 cell and M1 macrophages [103]. Proinflammatory immune cells produce high amounts of inflammatory cytokines such as TNF- α , IL-6, and IL-1 β and adipocytes produce high amounts of leptin, which promotes Th1 cells and inhibits Treg cells [103]. Adipose tissue inflammation by Th1/Th2 imbalance contributes to adipocyte hypertrophy and obesity-induced insulin resistance [104].

Probiotics are known to maintain a balance of Th1/Th2 immune at the level of receptor-ligand interactions by modulating immune response with bacterial cell wall components [105–107]. *Lactobacillus* and *Bifidobacterium* are Gram-positive bacteria whose cell wall is comprised of lipoprotein (LP) and lipoteichoic acid (LTA) embedded in thick layer of peptidoglycan (PGN) [108]. Previous study reported that strains in the genus *Lactobacillus* or *Bifidobacterium* induce the production of opposite cytokines IL-12 or IL-10, but combinational use of *Lactobacillus* and *Bifidobacterium* induced

the production of IL-10 and promoted Treg cell polarizing through interaction of LP and LTA with TLR2 [109]. It is previously reported that currently used two strains, *L. plantarum* CBT LP3 and *B. breve* CBT BR3, have an anti-inflammatory effect by increasing IL-10 and Treg cell and decreasing IL-6 and TNF- α [86, 90]. Therefore, it is assumed that *L. plantarum* CBT LP3 and *B. breve* CBT may modulate Th1/Th2 balance and adipose tissue inflammation. Although this study did not directly confirm whether immune regulation of current probiotics strains affected to the reduction of obesity symptoms, we suggested Th1/Th2 balance recovery by current probiotics as a possible mechanism.

Although probiotics are believed to help the activation of host immune system and to maintain intestinal barrier function, there can be some risks of using probiotics. Risks associated with probiotics treatment include minor gastrointestinal discomfort such as gas and bloating. In rare cases, probiotics can cause infections but cases of infection due to *Lactobacillus* and *Bifidobacterium* are extremely rare in immunocompromised subjects [110]. In our study, only one patient reported mild dyspepsia and diarrhea, and there was no sign for immunological problems. So far, concerns for immune deviation or excessive immune stimulation caused by probiotics are particularly relevant in the field of pregnant women and neonates who are vulnerable in immune-related diseases [111]. Currently, it is impossible to propose risk of autoimmune diseases and who is likely to have side effects. Since it is difficult to know which probiotics strains are suitable for each individual, dividing people by enterotype can be one strategy for prevention of autoimmune diseases after long-term probiotic consumption.

Enterotype as a potential microbiome biomarker

Recognizing that physicians should consider individual variability, precision medicine has shifted the focus on clinical trials from thousands-people to single-person measurement, so called N-of-1 trial [112]. True personalized medicine is based on the individual patient profile [113]. Although studies on individual genetic variation have been conducted to increase treatment efficiency, genetic variation alone is not sufficient to account for individual differences [114]. Considering meta-analysis studies, it is difficult to derive irrefutable conclusions about the anti-obesity effects of probiotics in clinical trials [115–117]. In clinical studies, various factors, such as diet, ethnicity, and individual differences in gut microbiome profiles, make it difficult to acquire consistent results from probiotics intake [84, 115]. To minimize individual differences and obtain effective results, it is necessary to evaluate by enterotypes to consider the subjects' gut microbiome profile.

Enterotype is influenced by diverse factors such as heredity and living environment. Among them, it is mostly affected by

food, and enterotypes are not easily altered by short-term dietary changes [42]. Advances in gut microbiome study have found that different metabolic responses appear with the same treatment depending on the enterotype [118, 119], and stratified analysis per enterotype is proposed for obesity management [120]. In this study, we identified positive responses of obesity-related parameters in the *Prevotella*-rich enterotype. Visceral fat showed a significant decrease in both enterotypes but a much larger decline in the *Prevotella*-rich enterotype than in *Bacteroides*-rich enterotype. Additionally, the *Prevotella*-rich enterotype showed decreased TG and total fat area after administering probiotics, while blood glucose, insulin, and fat ratios increased in the placebo group. Consistently in other studies, the *Prevotella*-rich enterotype exhibited an effective response to weight loss [47, 48]. However, other studies have shown that acarbose, an anti-diabetic drug, and capsaicin, an active compounds of chili pepper, improved metabolic parameters better in the *Bacteroides*-rich enterotype than in the *Prevotella*-rich enterotype [119, 121]. In our study, the inflammation level, C-reactive protein, was elevated in the placebo group of the *Bacteroides*-rich enterotype. It may be appear that the specific enterotype was not sensitive to response, but different enterotypes react according to different treatments. Due to the small sample size of our study, it was difficult to subdivide the enterotypes. However, in addition to the enterotypes represented by *Prevotella* and *Bacteroides*, enterotypes represented by *Ruminococcus* or *Enterobacteriaceae* have also been reported [43, 44], so detailed analysis of the enterotype in various and large-scale models is necessary.

Although we have found that probiotics can have a beneficial effect on obese subjects with *Prevotella*-rich enterotype, our result showed rather opposite results within placebo group. Subjects with *Prevotella*-rich enterotype showed increased glucose and insulin, while subjects with *Bacteroides*-rich enterotype did not show significant changes in glucose homeostasis. Because the placebo capsules we used in this study contain FOS, it might have affected the results itself. Both *Prevotella* and *Bacteroides* are well known as fiber-utilizing bacteria, and that the dominance of *Prevotella* or *Bacteroides* can differentially impact short-chain fatty acid (SCFA) productions. There was a study comparing fiber-utilizing capacity of *Prevotella* and *Bacteroides* with different fibers including FOS and they showed *Prevotella*-dominated microbiota produces more SCFA than *Bacteroides*-dominated microbiota [122]. Although it is well known that SCFA produced by microbiome have important effects on glucose homeostasis by range of mechanisms including improvement of gut barrier function and improved pancreatic β -cell function [123, 124], our result shows that just because there are a lot of SCFA-producing microbiota does not directly result in lowering the blood glucose and insulin levels. Moreover, our results may emphasize the importance of probiotics that it is better to take the prebiotics with the probiotics even if the subjects have abundant SCFA-producing bacteria in their gut.

Predictive, preventive, and personalized medicine

The recent progress in predictive, preventive, and personalized medicine (PPPM) has led to a paradigm shift from the uniform treatment for each disease to optimal health care taking account the characteristics of each patient [113]. Great potential for application of microbiome has been studied and implemented in personalized diets and disease prevention areas [125–127]. Enterotype classification speculates the reactivity and provides a personalized treatment for the individuals through the differential responses to drugs and diets [128–130]. We suggested that *Lactobacillus* and *Bifidobacterium* strains might be a supplementary therapy for preventing obesity in specific enterotype. Development of next probiotics for each enterotype is necessary to prevent obesity in personalized approach. Moreover, enterotype is potential microbiome biomarker. Enterotype-specific responses to the probiotics can be useful for predicting probiotics effect and developing personalized probiotics in predictive medicine approach. Obesity-related markers based on enterotype can help to stratify patients with obesity by predicting clinically relevant developments and facilitate to personalized individualized treatments and preventive measures. Enterotype-based PPPM might be applicable to the prevention and treatment of various diseases as well as obesity.

Study limitations

Some limitations should be addressed to interpret the findings of this study. Firstly, because our study aims to evaluate effect of GP2 on general adult population with obesity, the inclusion criteria was broad. It is well known that gender, age, ethnicity, and menopausal status can affect obesity prevalence as well as composition of gut microbiome [131–133]. To minimize confounding factors that influence both obesity and gut microbiota, individual clinical studies by age and gender groups are needed. Secondly, there may be limitations in the verification of the precise effects of probiotics strains. When making probiotics commercially, prebiotics such as FOS are usually added. We also add FOS when we make placebo capsules, so we can exclude bias by prebiotics. However, we think we should have performed clinical trials with probiotics with only strains in order to verify the effect of GP2. Thirdly, the study did not explain which pathways are involved with the effects. We suggested that the immune system plays an important role in anti-obesity effect of probiotics. Further researches of the molecular mechanisms underlying the effects of probiotics are needed to clarify the effects of probiotics and provide guidance for probiotic treatment as personalized and precision medicine.

Conclusions

In conclusion, this double-blinded, placebo-controlled, randomized clinical trial showed that GP2, a probiotic formulation containing *L. plantarum* and *B. breve*, changed obesity-related markers and biochemical markers. These changes differed per enterotype and were more pronounced in the *Prevotella*-rich enterotype than in the *Bacteroides*-rich enterotype. Nonetheless, we did not elucidate the exact mechanism for these enterotype-specific reactions. However, our study first reported a different response to the probiotics according to the enterotype, as well as the improvement of obesity-related markers by probiotics supplementation. Our findings provide important information for the use of probiotics to ameliorate the symptoms of obesity and for the application of probiotics in consideration of gut microbial enterotypes to treat obesity in a personalized way. In the future, gut microbial enterotype should be carefully examined to develop predictive diagnostics methods, targeted prevention, and personalized of medical services for obesity and other diseases.

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Compliance with ethical standards

Ethical approval This study was conducted according to the principles of Declaration of Helsinki and good clinical practice guidelines. The study protocol was approved by the Institutional Review Board of Ilsan Dongguk University Hospital (approval number 2016-02) and registered in Clinical Research Information Service (CRIS identifier: KCT0002292). Written informed consent has also been obtained from all the participants.

Competing interests The authors declare that they have no competing interests.

Consent for publication Not applicable.

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