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The triglyceride-lowering effect of supplementation with dual probiotic strains, *Lactobacillus curvatus* HY7601 and *Lactobacillus plantarum* KY1032: Reduction of fasting plasma lysophosphatidylcholines in nondiabetic and hypertriglyceridemic subjects

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Abstract *Background and aims:* This study evaluated the triglyceride (TG)-lowering effects of consuming dual probiotic strains of *Lactobacillus curvatus* (*L. curvatus*) HY7601 and *Lactobacillus plantarum* (*L. plantarum*) KY1032 on the fasting plasma metabolome.
Methods and results: A randomized, double-blind, placebo-controlled study was conducted on 92 participants with hypertriglyceridemia but without diabetes. Over a 12-week testing period, the probiotic group consumed 2 g of powder containing 5×10^9 colony-forming units (cfu) of *L. curvatus* HY7601 and 5×10^9 cfu of *L. plantarum* KY1032 each day, whereas the placebo group consumed the same product without probiotics. Fasting plasma metabolomes were profiled using UPLC-LTQ-Orbitrap MS. After 12 weeks of treatment, the probiotic group displayed a 20% reduction ($p = 0.001$) in serum TGs and 25% increases ($p = 0.001$) in apolipoprotein A-V (apoA-V). At the 12-week follow-up assessment, the following 11 plasma metabolites were significantly reduced in the probiotic group than the placebo group: palmitoleamide, palmitic amide, oleamide, and lysophosphatidyl choline (lysoPC) containing C14:0, C16:1, C16:0, C17:0, C18:3, C18:2, C18:1, and C20:3. In the probiotic group, changes (△) in TG were negatively correlated with △ apoA-V, which was positively correlated with △ FFA. In addition, △ FFA was strongly and positively correlated with △ lysoPCs in the probiotic group but not the placebo group.
Conclusions: The triglyceride-lowering effects of probiotic supplementation, partly through elevated apoA-V, in borderline to moderate hypertriglyceridemic subjects showed reductions in plasma metabolites, fatty acid primary amides and lysoPCs (NCT02215694; http://www.clinicaltrials.gov).
Clinical trials: NCT02215694; http://www.clinicaltrials.gov

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Introduction

Plasma triglycerides (TGs) are packaged into the TG-rich lipoproteins chylomicrons and very low-density lipoproteins (VLDL) that carry TGs from the diet or those that are synthesized in the liver, respectively. Elevated plasma TG levels and the prolonged circulation of lipoprotein remnants are independent risk factors for coronary artery disease [1–4]. A probiotic is defined as an effective regulation agent for the gut microflora. Over the past 10 years, probiotics have rapidly emerged as natural therapeutics with the potential to improve lipid metabolism [5–10]. For example, in cell cultures, *Lactobacillus plantarum* (*L. plantarum*) KY1032 induces the down-regulation of the genes involved in adipogenesis [11]. A recent study showed that a probiotic treatment of *Lactobacillus curvatus* (*L. curvatus*) HY7601 and *L. plantarum* KY1032 reduced average plasma TG levels by 40% compared with placebo treatment in rats on a high-fructose diet [10]. Therefore, we hypothesized that this effect might be extrapolated to humans, and we investigated the effect of supplementation with the dual probiotic strains *L. curvatus* HY7601 at 5×10^9 colony-forming units per day (cfu/d) and *L. plantarum* KY1032 at 5×10^9 cfu/d on their ability to lower TG in participants with mild-to-moderate hypertriglyceridemia (150–499 mg/dL). Specifically, this study aimed to determine the TG-lowering effects of probiotic supplementation on plasma metabolites. We also analyzed the plasma concentration of apolipoprotein A-V (apoA-V), which is a significant modulator of serum TG [12].

Methods

Participants and sample size determination

Participants were recruited from the Health Service Center (HSC) during routine check-ups at the National Health Insurance Corporation of Ilsan Hospital, Goyang, Korea from June 2012 to March 2014. Based on the data obtained from the HSC, participants with borderline-to-moderate hypertriglyceridemia were referred to the Departments of Family Medicine or Internal Medicine. The health and lipid profiles of these participants were re-evaluated; those who met the study criteria were invited to participate in a 12-week intervention study. Those who consented to the program were included in this study. Hypertriglyceridemia was defined based on the National Cholesterol Education Program (NCEP) – Adult Treatment Panel (ATP) III [13]. Participants with borderline-to-moderate hypertriglyceridemia but without diabetes were enrolled in the study after obtaining written consent. The IRB of Yonsei University and the National Health Insurance Corporation of Ilsan Hospital approved the study protocol. Participants were excluded if they used any medications or supplements known to affect lipid metabolism, any probiotic products over the past month or were diagnosed with dyslipidemia, diabetes mellitus, hypertension, liver disease, renal disease, cardiovascular disease, cerebrovascular disease, pancreatitis, cancer, or drug or alcohol abuse. Women who were pregnant or breastfeeding were also excluded. Study participants were randomly assigned to the probiotic or placebo groups. Computer-generated block randomization was employed for group assignment (probiotic:placebo = 1:1). Sample size was determined and calculated using SAS software package. In an exploratory clinical trial, the blood TG level of the intervention group decreased 62.33 ± 72.3 mg/dL (mean ± standard deviation) compared with the placebo group, showing little change in blood TG level. The sample size for this study was determined using the following parameters:

(1) Superiority test
(2) Level of significance, $\alpha = 0.025$
(3) $\beta = 0.05$, power = 95%
(4) Rate for participant number of probiotic product and placebo product, $\lambda = 1$, n_t (participant number of probiotic product) = λn_c (participant number of placebo product)
(5) We hypothesized that the change from probiotic products (μ_t) would be 62.33 mg/dL and the change from placebo (μ_c) would be 0 mg/dL.
(6) We supposed a standard deviation of 72.3 for both the probiotic (σ_t) and placebo (σ_c) groups.

$$H_0 : \mu_t \leq \mu_c$$

$$H_1 : \mu_t > \mu_c$$

The sample size was 35 participants per group.

$$n = \frac{(\sigma_t^2 + \sigma_c^2)(Z_{1-\alpha} + Z_{1-\beta})^2}{(\mu_t - \mu_c)^2} = 35$$

Assuming a dropout rate of 40%, 58 participants should be recruited per group.

$$n = \frac{n_t}{1 - r} = 58$$

Thus, 116 eligible participants were recruited for participation. A total of 24 participants dropped out of the study. Finally, 92 participants (probiotic group [$n = 46$], placebo group [$n = 46$]) completed the 12-week intervention study. Of the 92 participants, 64 had TG levels between 150 and 199 mg/dL; 26 had TG levels between 200 and 399 mg/dL; and 2 had TG levels between 400 and 499 mg/dL.

Study design and intervention

A 12-week, randomized, double-blind, placebo-controlled study was conducted with 92 participants with hypertriglyceridemia but without diabetes divided into two groups: a probiotic group ($n = 46$ individuals who consumed 2 g of powder that included dual probiotic strains containing *L. curvatus* HY7601 and *L. plantarum*

KY1032 at 5×10^9 cfu/d and 5×10^9 cfu/d, respectively, each day) and a placebo group ($n = 46$ individuals who consumed a powder that did not contain any probiotics each day). [NCT02215694; http://www.clinicaltrials.gov]. The 2 g of probiotic powder contained 0.2 g of *L. curvatus* HY7601, 0.2 g of *L. plantarum* KY1032, 1.14 g of crystalline cellulose, 0.4 g of lactose, and 0.06 g of a blueberry-flavoring agent. The 2 g of placebo powder contained 1.34 g of crystalline cellulose, 0.6 g of lactose, and 0.06 g of a blueberry-flavoring agent. Korea Yakult Co., Ltd (Seochogu, Seoul, Korea) provided the powders. The study consisted of two periods. The first included a screening period through which participants were identified as non-diabetic and hypertriglyceridemic via blood tests after not having ingested any probiotics for 14 days. The second period was an intervention period in which the participants ingested the probiotic or placebo powders each day for 12 weeks.

Anthropometric parameters, blood pressure (BP) and blood collection

Weight and height were measured when the participants were without clothes and shoes, and body mass index (BMI) was calculated (kg/m^2). Waist and hip circumferences were measured using a plastic measuring tape; these data were used to calculate the waist-to-hip ratio (WHR). BP was measured twice in the left arm of seated participants using an automatic BP monitor (FT-200S, Jawon Medical, Gyeongsan, Korea). After a 12-h fasting period, venous blood specimens were collected in EDTA-treated whole blood tubes and serum tubes. The blood samples were centrifuged to obtain plasma and serum. The collected samples were stored at −70 °C.

Serum lipid profile, free fatty acids (FFAs) and plasma apoA-V

Fasting TG and total cholesterol were measured using a Hitachi 7600 Autoanalyzer (Hitachi Ltd., Tokyo, Japan). HDL-cholesterol retained in the supernatant fraction was measured using an enzymatic method after lipoprotein precipitation. LDL-cholesterol was indirectly calculated using the Friedwald formula; LDL-cholesterol = total cholesterol − (HDL-cholesterol + [TG/5]) for participants with serum TG levels < 400 mg/dL. LDL-cholesterol was measured directly using a Hitachi 7600 Autoanalyzer for participants with serum TG levels ≥400 mg/dL. All participants ingested 75 g of glucose solution after a 12-h overnight fast. Venous specimens were collected before glucose loading, at loading, and at 30, 60, and 120 min after loading to determine TG response. FFAs were measured using an enzymatic assay with the acyl-CoA synthetase-acyl-CoA oxidase method using a Hitachi 7600 Autoanalyzer. Plasma apoA-V was measured using an enzyme immunoassay (Human Apolipoprotein A ELISA kit, Millipore, MO), and the color reaction was monitored at 450 nm using a Victor^2 spectrometer (Perkin Elmer Life Sciences, Turka, Finland). The intra- and inter-assay coefficients of variance (%CV) were 9.06% and 7.56%, respectively.

Fasting serum glucose, insulin, homeostasis-model assessment of insulin resistance (IR), C-peptide, plasma oxidized LDL, lipoprotein-associated phospholipase A_2 activity and high-sensitivity C-reactive protein (hs-CRP)

Fasting serum glucose was measured using the hexokinase method with a Hitachi 7600 Autoanalyzer. Serum insulin was measured using an immunoradiometric assay kit from DIAsource ImmunoAssays S.A. (Louvain, Belgium). IR was calculated using the homeostasis-model assessment (HOMA) via the following equation: HOMA-IR = (Fasting insulin [μIU/mL] × Fasting glucose [mmol/L])/22.5. Serum C-peptide was measured using an immunoradiometric assay with a C-peptide IRMA kit (Immunotech, Czech). Plasma oxidized LDL (ox-LDL) was measured using an enzyme immunoassay (Mercodia, Uppsala, Sweden). The resulting color reaction was monitored at 450 nm with a Wallac Victor^2 multilabel counter (Perkin Elmer Life Sciences, Turku, Finland). The intra- and inter-assay %CVs were 2.29% and 3.30%, respectively. The activity of the lipoprotein-associated phospholipase A_2 (Lp-PLA_2) was measured using a high-throughput radiometric activity assay. The intra- and inter-assay %CVs were 7.37% and 12.6%, respectively. Serum hs-CRP was measured using an ADVIA® 2400 Clinical Chemistry System (Siemens Ltd., Tarrytown, NY) and a high-sensitivity CRP-Latex(II) X2 kit (Denka-Seiken Co., Ltd., Tokyo, Japan).

Global (non-targeted) metabolic profiling of plasma

Sample preparation and analysis

Prior to analysis, 800 μL of 80% acetonitrile was added to 100 μL of plasma, mixed by vortexing, and centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was dried with N_2, dissolved in 10% methanol, mixed by vortexing, and centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was transferred to a vial.

Ultra performance liquid chromatography (UPLC)

The plasma extract samples (4 μL) were injected into an Acquity UPLC-BEH-C18 column (2.1 × 50 mm, 1.7 μm; Waters, Milford, MA) that was coupled in-line with a UPLC-LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, MA). The injected samples were equilibrated with water containing 0.1% formic acid. Samples were eluted with an acetonitrile gradient containing 0.1% formic acid at a flow rate of 0.35 mL/min for 20 min. Metabolites were separated via UPLC (Thermo Fisher Scientific, Waltham, MA), analyzed, and assigned using LTQ-Orbitrap-XL (Thermo Fisher Scientific, Waltham, MA). The mass spectrometer was operated in electrospray ionization (ESI)-positive mode, and the spray voltage was 5 kV. The flow rate of the nitrogen sheath gas and the auxiliary gas were 50 and 5 (arbitrary units), respectively. The capillary voltage, tube-lens voltage, and capillary temperature were kept constant at 35 V, 80 V, and 370 °C, respectively. The Orbitrap data

were collected in the range of m/z 50−1000. For quality control, a mixture of four standard compounds (acetaminophen, sulfadimethoxine, terfenadine, and reserpine) was injected every ten samples. The MS/MS spectra of metabolites were obtained using a collision-energy ramp from 55 to 65 eV conducted with Xcalibur 2.1 and MS Frontier software (Thermo Fisher Scientific, Waltham, MA).

Data processing and identification of metabolites

All MS data including retention times, m/z, and ion intensities were extracted using SIEVE software (Thermo Fisher Scientific, Waltham, MA) and incorporated into the instrument. The resulting MS data were assembled into a matrix. The SIEVE parameters were set as follows: m/z range 50−1000; m/z width = 0.02; retention time width = 2.5; m/z tolerance = 0.005. Metabolites were searched for in the following databases: ChemSpider (www.chemspider.com), Human Metabolome (www.hmdb.ca), Lipid MAPS (www.lipidmaps.org), KEGG (www.genome.jp/kegg), and MassBank (www.massbank.jp). Selected metabolites were confirmed using standards based on both retention times and mass spectra.

Assessment of food intake and physical activity

The usual dietary intake of the participants was assessed using a semi-quantitative food frequency questionnaire and the 24-h recall method. Nutrient intake was determined and calculated based on 3-day food records using the Computer-Aided Nutritional Analysis Program (CAN-pro 3.0; Korean Nutrition Society, Seoul, Korea). Total energy expenditure (kcal per day) was calculated based on the activity patterns of the participants including their basal metabolic rate (BMR), 24-h physical activity level, and the specific dynamic actions of food. The BMR for each participant was calculated using the Harris−Benedict equation.

Statistical analyses

Statistical analyses were performed using SPSS v. 21.0 (IBM SPSS Statistics 21, Armonk, NY, USA). Skewed variables were logarithmically transformed for analysis. For descriptive purposes, mean values are presented using untransformed values. Results are expressed as means ± standard deviations (SDs) and medians (interquartile ranges; IQRs). A two-tailed p-value of <0.05 was considered significant. An independent t-test was used to compare parameters between the placebo and probiotics groups. A general linear model was applied to adjust for potential confounds. A paired t-test was used to evaluate the differences between baseline and the 12-week follow-up levels for each group. Pearson's correlation coefficient was used to examine the relationships between variables. Multiple regression analyses were performed to identify the major plasma metabolites associated with the blood lipid profile. False discovery rate corrected q-values were computed using the R package "fdrtool". A heat map was generated to visualize correlations among variables.

Multivariate statistical analyses were performed using SIMCA-P+ version 12.0 (Umetrics, Umeå, Sweden). A partial least-squares discriminant analysis (PLS-DA) was used as the classification method for modeling the between-group discrimination by visualizing the score plot (S-plot) using the first and second PLS components. To validate the PLS-DA model, a seven-fold validation was applied, and the reliabilities of the model were rigorously validated via a permutation test ($n = 200$). Goodness of the fit was quantified using R^2Y, whereas predictive ability was quantified using Q^2Y. In general, R^2Y describes how well the data in the training set are mathematically reproduced, and this value varies between 0 and 1, where a value of 1 indicates a model with a perfect fit. Models in which $Q^2Y \geq 0.5$ are considered to have satisfactory predictive capabilities.

Results

The effects of consuming probiotics for 12 weeks with regard to clinical and biochemical characteristics

Table 1 shows the general and biochemical characteristics of the placebo and probiotic groups. No significant between-group differences were observed at baseline with regard to age, gender distribution, smoking, drinking, BMI, BP, serum lipid profiles, ox-LDL, Lp-PLA_2 activity, hs-CRP, apoA-V, glucose, insulin, HOMA-IR, or C-peptide. Estimated total calorie intake, physical activity, % protein intake, % fat intake, and % carbohydrate intake did not significantly differ between groups (data not shown). After 12 weeks of treatment, individuals in the probiotic group exhibited a 20% reduction ($p = 0.001$) in their serum TG levels and a significant 25% increase ($p = 0.001$) in their plasma apoA-V levels. The probiotic group also showed significant increases in FFAs. However, the placebo group did not exhibit significant changes.

Plasma metabolic profiling using UPLC-LTQ-Orbitrap MS

Non-targeted metabolic pattern analysis

The MS data of the plasma metabolites obtained at baseline and the 12-week follow-up assessment were analyzed using a PLS-DA score scatterplot. PLS-DAs for the MS data of the plasma metabolites were conducted for the following three combinations of groups: (1) placebo at baseline and the 12-week follow-up assessment (Fig. 1a); (2) probiotics at baseline and at 12-week follow-up assessment (Fig. 1b); and (3) placebo at the 12-week follow-up assessment and probiotics at the 12-week follow-up assessment. The two-component PLS-DA scatterplots of the plasma metabolites for the first combination of groups did not show distinct clustering or a clear separation of participants in the placebo group at baseline or the 12-week follow-up assessment (R^2X[cum] = 0.134, R^2Y[cum] = 0.675, Q^2Y[cum] = 0.184; Fig. 1a). The PLS-DA model was validated using a permutation test, which indicated an R^2Y intercept value of 0.532 and a Q^2Y intercept value of −0.044. The two-component PLS-DA scatterplots of the plasma metabolites for the second

Table 1 Clinical and biochemical characteristics of the placebo and probiotic groups at baseline and the 12-week follow-up assessment.

	Total (n = 92)				p^a	p^b	p^c	p^d
	Placebo group (n = 46)		Probiotic group (n = 46)					
	Baseline	Follow-up	Baseline	Follow-up				
Age (year)	52.7 ± 8.78		54.1 ± 7.90		0.412			
Male/Female n, (%)	15 (30.4)/31(69.6)		15 (32.6)/31 (67.4)		0.822			
Cigarette smoker n, (%)	5 (10.9)		6 (13.0)		0.748			
Alcohol drinker n, (%)	24 (52.2)		27 (58.7)		0.529			
Body mass index (kg/m^2)	24.9 ± 2.26	24.9 ± 2.36	24.7 ± 2.91	24.6 ± 2.97	0.777	0.675		
Systolic BP (mmHg)	121.9 ± 13.5	122.8 ± 15.4	120.4 ± 15.3	118.8 ± 11.5	0.601	0.157		
Diastolic BP (mmHg)	79.3 ± 8.32	80.8 ± 10.5	78.3 ± 10.4	78.0 ± 8.66	0.597	0.160		
Total-cholesterol (mg/dL)$^\phi$	201.8 ± 31.0	201.7 ± 33.8	210.5 ± 26.8	214.7 ± 29.2	0.129	0.254		
HDL-cholesterol (mg/dL)$^\phi$	45.3 ± 7.06	46.0 ± 8.06	46.0 ± 7.57	47.4 ± 8.98	0.674	0.440		
LDL-cholesterol (mg/dL)$^\phi$	115.1 ± 29.5	115.9 ± 32.7	125.1 ± 29.1	127.7 ± 26.6	0.111	0.047		
Triglyceride (mg/dL)$^\phi$	207.0 ± 45.8	225.4 ± 118.3	210.2 ± 77.2	167.9 ± 61.9**	0.853	0.008		
Change	18.4 ± 122.5		−42.3 ± 93.9				0.009	0.002
Free fatty acid (μEq/L)$^\phi$	492.9 ± 171.5	493.0 ± 166.9	523.0 ± 173.9	588.0 ± 153.9*	0.306	0.003		
Oxidized LDL (U/L)$^\phi$	48.1 ± 15.9	48.1 ± 13.3	47.9 ± 9.54	51.0 ± 11.1*	0.654	0.137		
Lp-PLA$_2$ activity (nmol/mL/min)$^\phi$	22.1 ± 8.42	21.5 ± 8.79	25.6 ± 7.75	25.8 ± 8.21	0.325	0.104		
hs-CRP (mg/dL)$^\phi$	1.14 ± 0.99	1.50 ± 2.00	1.25 ± 1.13	1.66 ± 2.04	0.941	0.593		
Apolipoprotein A-V (ng/mL)$^\phi$	180.7 ± 143.1	191.8 ± 163.0	193.2 ± 131.2	240.8 ± 147.1**	0.356	0.016		
Change	11.1 ± 83.7		47.6 ± 80.4				0.036	0.032
Glucose (mg/dL)$^\phi$	90.6 ± 14.6	91.8 ± 15.2	89.9 ± 12.2	91.4 ± 13.7	0.900	0.935		
Insulin (μIU/dL)$^\phi$	9.66 ± 3.51	9.24 ± 3.56	9.19 ± 3.22	9.94 ± 3.77	0.536	0.274		
HOMA-IR$^\phi$	2.17 ± 0.89	2.10 ± 0.91	2.02 ± 0.71	2.25 ± 0.97	0.523	0.327		
C-peptide (μEq/L)$^\phi$	2.18 ± 0.69	2.14 ± 0.70	2.08 ± 0.86	2.20 ± 0.68*	0.309	0.604		

Mean ± SD. $^\phi$tested after logarithmic transformation; p^a-values derived from independent *t*-test at baseline; p^b-values derived from independent-samples *t*-tests at follow up; p^c-values derived from independent-samples *t*-tests for the changed value; p^d-values adjusted for baseline; $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ derived from paired-samples *t*-tests.

combination of groups showed distinct clustering and clear separation for the participants in the probiotics group at baseline and the 12-week follow-up assessment (R^2X[cum] = 0.167, R^2Y[cum] = 0.761, Q^2Y[cum] = 0.611; Fig. 1b). The PLS-DA model was validated using a permutation test, which indicated an R^2Y intercept value of 0.518 and a Q^2Y intercept value of −0.205. The two-component PLS-DA scatterplots of the plasma metabolites for the third combination of groups also showed distinct clustering and clear separation for the participants who used probiotics at the 12-week follow-up assessment and those who received the placebo at the 12-week follow-up assessment (R^2X[cum] = 0.184, R^2Y[cum] = 0.742, Q^2Y[cum] = 0.573; Fig. 1c). The PLS-DA model was validated using a permutation test, which indicated an R^2Y intercept value of 0.535 and a Q^2Y intercept value of −0.136. These results clearly show that plasma metabolomic profiles can distinguish groups based on probiotics, changes in probiotics-induced biochemical characteristics, or both. To identify the metabolites that differentially determined the data at baseline and the 12-week follow-up assessment, *S*-plots of *p*(1) and *p(corr)* (1) were generated using centroid scaling (Fig. 1d, e, f). The *S*-plots revealed that metabolites with higher or lower *p(corr)* values were most relevant for discriminating between the two groups.

Identification of plasma metabolites

Of the 1898 plasma metabolites, those that played important roles in separating the groups were selected according to the Variable Important in the Projection (VIP) parameter; VIP values > 1.0 indicate a high relevance for the difference between the sample groups. A total of 113 metabolites were identified with VIP values >1.0; 21 of these metabolites were previously identified, and 92 metabolites were unknown. The results are shown in Table 2. No significant differences in the baseline metabolites were observed between the placebo and probiotic groups. At the 12-week follow-up assessment, the placebo group did not show any significant changes for any of the 21 metabolites, whereas the probiotic group showed the following plasma metabolite changes: 16 metabolites showed significant decreases (N-ethyldodecamide, N-propyldodecamide, plamitoleamide, palmitic amide, oleamide, lactosyceramide, and lysoPCs containing C14:0, C16:1, C16:0, C17:0, C18:3, C18:2, C18:1, C20:5, C20:3 and C22:6) (Table 2). Next, we compared the plasma metabolite changes between the placebo and probiotic groups. The probiotic group showed greater reductions in palmitoleamide (q = 0.025), palmitic amide (q = 0.011), oleamide (q = 0.031), lysophosphatidyl choline (lysoPC; 14:0; q = 0.040), lysoPC (16:1; q = 0.018), lysoPC (16:0; q = 0.029), lysoPC (17:0; q = 0.046), lysoPC (18:3; q = 0.030), lysoPC (18:2; q = 0.044), lysoPC (18:1; q = 0.011), and lysoPC (20:3; q = 0.036; Table 2). At the 12-week follow-up assessment, the probiotic group showed lower peak intensities of N-propyldodecamide, plamitoleamide, palmitic amide, oleamide, and lysoPCs containing C14:0, C16:1, C16:0, C18:3, C18:2, C18:1, and C20:3 (Table 2).

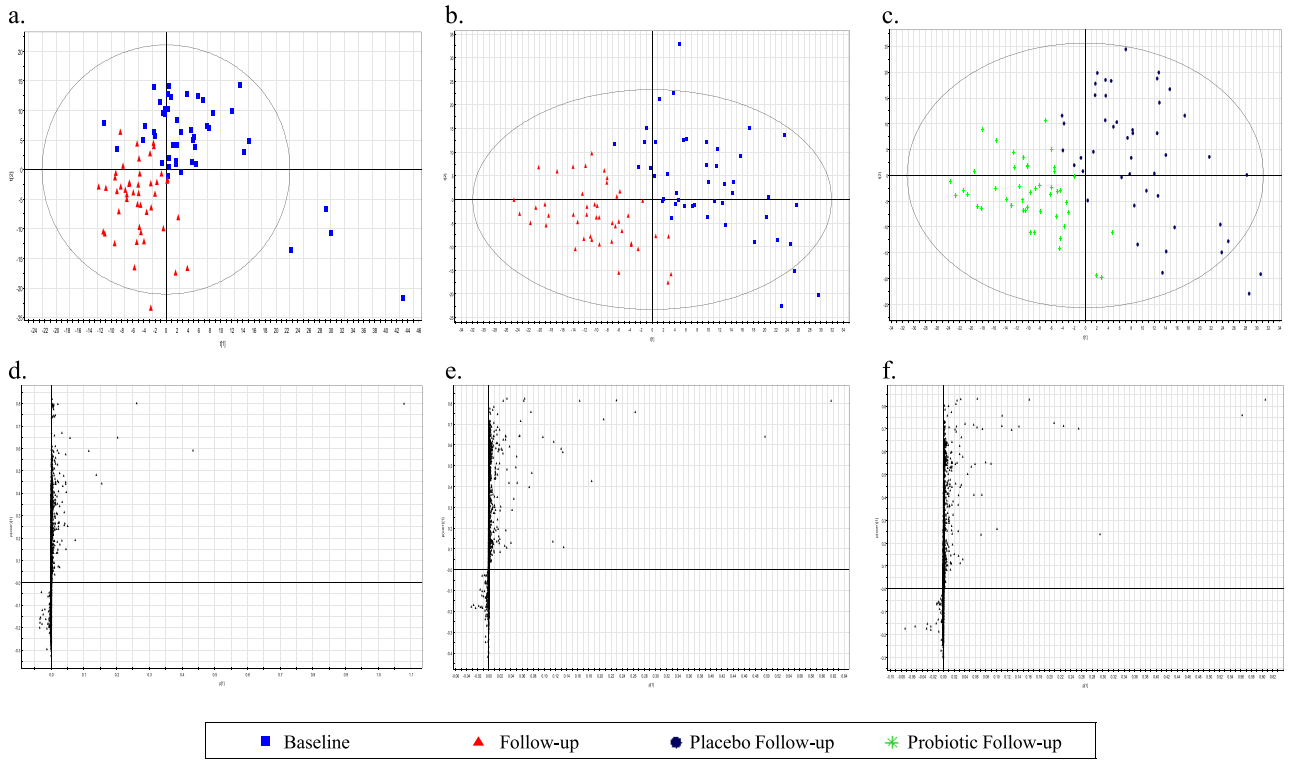

Figure 1 Identification of significantly altered plasma metabolites at the 12-week follow-up assessment. a. *S*-plots from the PLS-DA models for the placebo group (n = 46) at baseline and the 12-week follow-up assessment. b. *S*-plots from the PLS-DA models for the probiotic group (n = 46) at baseline and the 12-week follow-up assessment. c. *S*-plots from PLS-DA models for the placebo and probiotic groups at the 12-week follow-up assessment. d, e, f. *S*-plots for co-variance (p) and reliability correlation (p[corr]) from the PLS-DA models.

Relationships among the changes in TG, apoA-V, FFA, and major plasma metabolites

The correlation between the changed levels (△) of TG, apoA-V, FFA, and major plasma metabolites were determined after adjusting for changes in age, gender, and BMI in the placebo and probiotic groups (Fig. 2). In the placebo group, △ TG was positively correlated with △ lysoPC (14:0), whereas △ FFA was negatively correlated with △ tryptophan and △ docosanamide. In the probiotic group, however, △ TG was negatively correlated with △ apoA-V ($r = -0.472$, $p = 0.001$, $q = 0.020$), and △ apoA-V was positively correlated with △ FFA ($r = 0.325$, $p = 0.033$, $q = 0.046$). Furthermore, △ FFA in the probiotic group was negatively correlated with △ proline betaine, △ N-ethyldodecamide, △ N-propyldodecamide, △ plamitoleamide, △ palmitic amide, and △ oleamide but strongly and positively correlated with △ lysoPCs containing C16:1, C16:0, C17:0, C18:1, C20:3, and C22:6 (Fig. 2). The *q*-values for the significant associations were satisfactory ($4.9E\text{-}5 \leq q \leq 0.051$).

Discussion

Because the dual probiotic strains *L. curvatus* HY7601 and *L. plantarum* KY1032 reduced the average plasma TG levels of animals by 40% compared with a placebo treatment [10], we hypothesized that this finding might be extrapolated to humans. In this 12-week follow-up study, daily supplementation with a combination of *L. curvatus* HY7601 and *L. plantarum* KY1032 delivered as a 2 g powder to participants with mild-to-moderate hypertriglyceridemia but without diabetes led to a significant 20% reduction in serum TG and a significant 25% increase in plasma apoA-V levels. Furthermore, considering the probiotics-related alterations in plasma metabolites, we observed a lowering effect of the probiotic strains on specific metabolites, with greater reductions in the probiotic group than the placebo group for 3 fatty acid primary amides including palmitoleamide, palmitic amide, and oleamide as well as 8 lysoPCs containing C14:0, C16:1, C16:0, C17:0, C18:3, C18:2, C18:1, and C20:3. These significant reductions in plasma lysoPCs and fatty acid primary amides might be a direct effect of probiotics on plasma metabolites, or they might be effects of other probiotic-induced biochemical changes, particularly circulating levels of TG and apoA-V.

Plasma TG concentrations are the result of the balance between TG-rich lipoprotein secretion by the intestine and liver and its uptake by the extra-hepatic tissues through lipoprotein lipase (LPL), which catalyzes the hydrolytic cleavage of TG into fatty acids [14,15]. ApoA-V is implicated in TG metabolism [14] and might be a potent factor affecting plasma TGs in human. The function of apoA-V in accelerating plasma TG hydrolysis might be to enhance LPL activity; to increase the LPL concentrations at endothelial sites, stimulating LPL mediated hydrolysis; or to change

Table 2 Identification of plasma metabolites in the placebo and probiotic groups at baseline and the 12-week follow-up assessment.

Identity	Formula $[M+H]^+$	Exact Mass (M + H)	Normalized peak intensities				VIP		
			Placebo group (n = 46)		Probiotic group (n = 46)		Baseline vs. Follow-up		12-week
			Baseline	Follow-up	Baseline	Follow-up	Placebo	Probiotic	Placebo vs. Probiotic
L-Leucine	$C_6H_{13}NO_2$	132.1024	13,59,321 ± 2,66,218	12,81,324 ± 2,28,860	13,38,445 ± 2,72,452	12,81,497 ± 2,49,401	3.2521	0.9586	0.8240
Proline betaine	$C_7H_{13}NO_2$	144.1024	1,12,379 ± 1,05,263	1,06,376 ± 82,835	78,075 ± 61,778	1,06,886 ± 75,150	0.5630	1.1751	0.2295
L-Tryptophan	$C_7H_{13}NO_2$	205.0977	7,70,512 ± 1,68,269	7,31,926 ± 1,09,182	7,25,420 ± 1,70,691	6,99,054 ± 96,388	2.4771	0.6755	0.8835
N-ethyldodecanamide	$C_{14}H_{29}NO$	228.2327	3,61,684 ± 3,22,920	3,65,758 ± 2,53,931	3,75,779 ± 2,93,640	2,41,433 ± 2,23,524*	1.0598	2.9553	3.2778
N-propyldodecanamide	$C_{15}H_{31}NO$	242.2484	1,32,904 ± 1,22,466	1,36,401 ± 95,573	1,44,007 ± 1,22,167	83,620 ± 85,790*,†	0.3474	1.2481	1.3830
Palmitoleamide	$C_{16}H_{31}NO$	254.2484	5,19,419 ± 3,97,171	5,61,968 ± 3,00,103	5,92,321 ± 4,04,293	3,40,267 ± 2,60,594**,††	2.1330	5.1021	5.7920
Change			42,549 ± 4,74,494		−2,52,054 ± 4,43,957‡				
Palmitic amide	$C_{16}H_{33}NO$	256.2640	4,03,509 ± 2,84,903	4,45,024 ± 2,23,137	4,96,050 ± 3,47,509	266,424 ± 2,07,638***,††	2.1384	4.6361	4.6714
Change			41,515 ± 3,34,371		−2,29,626 ± 3,48,927‡				
Oleamide	$C_{18}H_{35}NO$	282.2797	22,91,503 ± 13,06,244	25,20,731 ± 10,87,512	26,61,203 ± 13,17,106	16,90,921 ± 9,85,609***,††	14.7154	19.6072	21.7140
Change			2,29,227 ± 15,59,571		−9,70,282 ± 15,12,645‡				
Docosanamide	$C_{22}H_{45}NO$	340.3579	1,39,161 ± 89,459	1,15,634 ± 66,079	1,21,401 ± 67,688	1,37,449 ± 1,08,385	1.0507	1.7238	1.4595
Cholesteryl acetate	$C_{29}H_{48}O_2$	429.3732	49,607 ± 65,689	6,09,230 ± 81,772	25,876 ± 40,906	29,192 ± 46,971	0.5105	0.2622	1.2439
LysoPC (14:0)	$C_{22}H_{46}NO_7P$	468.3090	2,31,345 ± 80,840	2,10,112 ± 67,509	2,36,278 ± 62,413	1,65,516 ± 71,769***,†	1.0312	1.5583	1.1673
Change			−21,233 ± 92,430		−70,762 ± 78,725‡				
LysoPC (16:1)	$C_{24}H_{48}NO_7P$	494.3246	4,22,435 ± 1,40,504	4,07,569 ± 1,64,155	4,43,786 ± 1,35,770	313,570 ± 1,11,273***,†	0.8312	2.8628	2.4840
Change			−14,866 ± 1,97,410		−1,30,215 ± 126873‡				
LysoPC (16:0)	$C_{24}H_{50}NO_7P$	496.3403	67,90,109 ± 13,07,247	64,43,010 ± 10,70,318	68,14,787 ± 11,59,336	55,80,381 ± 9,61,358***,††	15.5528	24.9018	22.6483
Change			−3,47,099 ± 16,33,741		−12,34,406 ± 9,61,358‡				
LysoPC (17:0)	$C_{25}H_{52}NO_7P$	510.3559	2,01,721 ± 81,521	1,81,608 ± 78,137	2,25,2,10 ± 80,661	1,58,605 ± 59,658***	0.9184	1.3958	0.7781
Change			−20,113 ± 93,150		−66,604 ± 72,237‡				
LysoPC (18:3)	$C_{26}H_{48}NO_7P$	518.3246	3,26,336 ± 80,485	3,26,147 ± 91,562	3,21,200 ± 76,099	2,71,227 ± 46,808***,††	0.6766	1.0153	1.4534
Change			−190 ± 85108		−49,973 ± 75,153‡				
LysoPC (18:2)	$C_{26}H_{50}NO_7P$	520.3403	23,84,519 ± 6,20,577	22,84,947 ± 5,16,828	23,55,835 ± 5,24,674	19,25,211 ± 5,18,466***,†	4.5018	10.2753	9.4535
Change			−99,572 ± 6,49,494		−4,30,624 ± 521463‡				
LysoPC (18:1)	$C_{26}H_{52}NO_7P$	522.3559	20,04,873 ± 4,97,487	19,18,557 ± 4,35,955	20,35,520 ± 4,33,681	1,5,65,052 ± 3,47,661***,††	3.9261	9.5167	9.2788
Change			−86,316 ± 5,18,012		−4,70,468 ± 4,45,430‡				
LysoPC (20:5)	$C_{28}H_{48}NO_7P$	542.3246	3,01,242 ± 1,33,617	2,71,395 ± 1,10,007	3,22,003 ± 1,56,385	2,60,372 ± 1,15,692**	1.4412	1.3850	0.9759
LysoPC (20:3)	$C_{30}H_{50}NO_7P$	546.3559	2,81,731 ± 96,409	262,595 ± 75,456	2,69,420 ± 74,184	1,97,215 ± 63,341***,††	0.8543	1.5948	1.7494
Change			−19,136 ± 1,05,522		−72,206 ± 69,789‡				
LysoPC (22:6)	$C_{28}H_{48}NO_7P$	568.3403	3,88,062 ± 1,59,660	3,31,323 ± 1,00,000	4,02,365 ± 1,39,771	3,10,312 ± 1,20,444***	2.9551	2.0933	1.0596
Lactosylceramide (d18:1/12:0)	$C_{42}H_{79}NO_{13}$	806.5629	4,92,370 ± 2,92,268	4,53,304 ± 2,18,168	4,92,083 ± 2,63,213	3,97,723 ± 1,97,735**	1.7874	2.0098	1.7379

Mean ± SD. $q^{*} < 0.05$, $q^{**} < 0.01$, $q^{***} < 0.001$ derived from paired-samples *t*-tests; $q^{\dagger} < 0.05$, $q^{\dagger\dagger} < 0.01$, $q^{\dagger\dagger\dagger} < 0.001$ derived from independent-samples *t*-test at follow up. $q^{\ddagger} < 0.05$, $q^{\ddagger\ddagger} < 0.01$, $q^{\ddagger\ddagger\ddagger} < 0.001$ derived from △ between placebo and probiotic group.

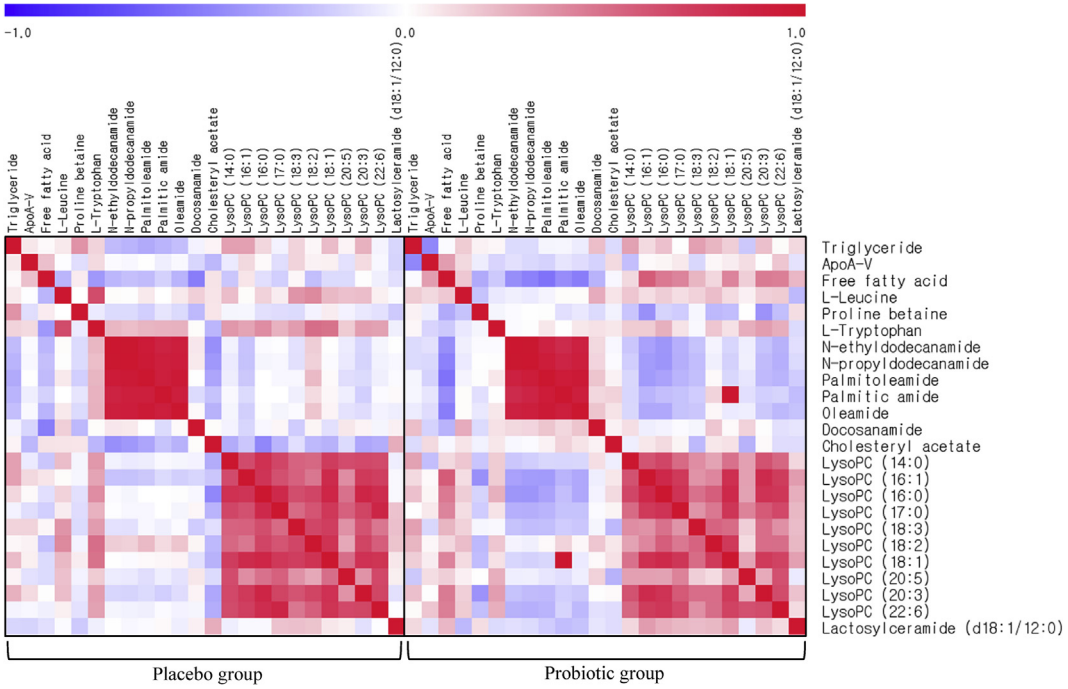

Figure 2 Correlation matrix among △ TG, △ apoA-V, △ FFA, and △ plasma metabolite in the placebo and probiotic groups. Correlations were obtained by calculating Spearman's correlation coefficient. Red denotes a positive correlation, and blue represents a negative correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the effects of LPL in regulating apolipoproteins [14]. Data collected from humans have not clearly indicated TG-lowering effects due to apoA-V, possibly because too many different genetic and environmental co-factors are needed to produce a hypertriglyceridemic phenotype. However, this study observed a negative correlation between △ TG and △ apoA-V in the probiotic group. In addition, the positive correlation between △ apoA-V and △ FFA in the probiotic group might support the primary mechanism for TG-lowering by apoA-V via the stimulation of LPL [14,15].

Interestingly, we also found that △ FFA in the probiotic group were strongly and positively correlated with △ lysoPCs containing C16:1, C16:0, C17:0, C18:1, C20:3, and C22:6, whereas this correlation was not observed in the placebo group. Phospholipase A_2 (PLA_2) enzymes, including lipoprotein-associated PLA_2 (Lp-PLA_2), hydrolyze the *sn-2* fatty acyl ester bond in the phosphatidyl cholines (PCs) of LDL, yielding FFAs and lysoPCs [16,17]. In addition, lecithin:cholesterol acyltransferase (LCAT) forms significant amounts of plasma lysoPCs [18]; LCAT catalyzes the transfer of fatty acids at position *sn-2* of the PCs to the free cholesterol in plasma, forming cholesterol esters and lysoPC. In the probiotic group of this study, however, no significant changes were observed in plasma Lp-PLA_2 activity or plasma cholesteryl esters (i.e., normal human cholesterol esters). Therefore, the significant decrease in plasma lysoPCs at 12 weeks compared with baseline in the probiotic group might reflect decreased circulating levels of TG-rich lipoproteins. This study identified plasma lysoPC 16:0 (VIP = 22.6483) and oleamide (VIP = 21.7140) as the most important plasma metabolites for evaluating the difference between placebo and probiotics groups at 12 weeks.

Although many studies demonstrate the beneficial effects of probiotics on the modulation of serum lipid levels [5–9], some studies have not observed any improvement in blood lipid profiles [19,20]. Furthermore, the mechanism that underlies the modulation of serum lipid profiles via probiotics remains unclear. In the probiotic group of this study, no significant change was observed in the circulating levels of insulin and glucose that play a role in the regulation of apoA-V gene expression [21–23]. Thus, factors other than insulin and glucose might be involved in increasing the circulating levels of apoA-V in the probiotic group. According to Park et al. [10], a 10^{10} cfu/d probiotic treatment (*L. curvatus* HY7601 and *L. plantarum* KY1032) for 3 weeks in rats with high-fructose-induced metabolic syndrome led to transcriptional factor expression changes that significantly reduced plasma TG levels. A hepatic gene expression analysis showed that probiotic supplementation in high-fructose-diet-fed rats up-regulated PPARα (+76%), and CPT2 (+66%) expression but down-regulated SREBP1 (−30%), FAS (−54%), and SCD1 (−23%) expression. The up-regulation of PPARα and CPT2 expression reflected the activation of fatty acid β-oxidation, and the down-regulation of SREBP1, FAS, and SCD1 expression reflected the suppression of lipogenesis [24–27]. Recent studies have shown that the up-regulation of PPARα expression increases apoA-V [28,29], and TG levels were reduced by the stimulation of apoA-V [30]. Therefore, probiotic supplementation might increase apoA-V and therefore decrease TG levels through the up-regulation of PPARα.

Recent reports of the potential mechanisms of the probiotic effects on lipid metabolism show (1) that the lipid-lowering effect of the probiotic is not because of a redistribution of lipids from the plasma to the liver (rather, it is attributed to a decrease in lipid intestinal absorption or an increase in lipid catabolism) [31,32]; and (2) the reduction of plasma TG level is correlated with the decrease of the TG in VLDL and remnant lipoproteins [33]. Additional investigations are needed to identify how the various responses, mechanisms, and relevant pathways related to lipid metabolism are influenced by various probiotics.

Recent studies have reported that gut microbiota are associated with metabolic phenotypes [34]. A metabolomics study reported that the gut microbiota-dependent metabolism of dietary lipid PCs were directly associated with the risk of cardiovascular disease (CVD). Gut microbiota play a role in metabolite production from dietary PC. The suppression of gut microbiota through inhibited dietary PC enhances atherosclerosis in mice [35]. In the present study, probiotic supplementation affected blood lipid profile and metabolites in plasma. These results indicate that the manipulation of gut microflora through use of probiotics have the potential to influence the metabolism of lipid-regulating pathways and prevent the onset of CVD.

Our study design has several limitations. First, dietary intake was based on self-reports obtained from weighed food. However, measurement errors from self-reported dietary intake and lifestyle variables are relatively small [36]. Second, we specifically focused on Korean participants with mild-to-moderate hypertriglyceridemia without diabetes. Therefore, our data cannot be generalized to other ethnic groups, patients with severely hypertriglyceridemia or those with normal blood TG levels. Finally, numerous metabolite markers were detected using UPLC-LTQ-Orbitrap MS in this study; however, most of these metabolites remain unidentified. Large databases of endogenous biomolecules have not yet been constructed for use with LC-MS-based techniques for metabolomics research [37]. Despite these limitations, the TG-lowering effects of probiotic supplementation, partly through elevated apoA-V, significantly reduced plasma metabolites including the fatty acid primary amides palmitoleamide, palmitic amide, oleamide as well as 8 lysoPCs containing C14:0, C16:1, C16:0, C17:0, C18:3, C18:2, C18:1, and C20:3.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.numecd.2015.05.002

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