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Article

Regulation of alcohol and acetaldehyde metabolism by a mixture of *Lactobacillus* and *Bifidobacterium* species in human

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Abstract: Excessive alcohol consumption is one of the significant causes of morbidity and mortality worldwide. Alcohol is oxidized to toxic and carcinogenic acetaldehyde by alcohol dehydrogenase (ADH) and further oxidized to a non-toxic acetate by aldehyde dehydrogen ase (ALDH). Emerging evidence shows that *Lactobacillus* and *Bifidobacterium* species encode alc ohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) mediate alcohol and acetaldehyde metabolism, respectively. This study involves supplementation of *Lactobacillus* a nd *Bifidobacterium probiotic mixture* in humans and assessed their effects on alcohol and aceta ldehyde metabolism. Here, twenty-seven wild types (ALDH2*1/*1) and the same number of heterozygotes (ALDH2*2/*1) were recruited for the study. The enrolled participants were ra ndomly divided into either the probiotic (Duolac ProAP4) or the placebo group. Each grou p received a probiotic or placebo capsule for 15 days with subsequent crossover. Primary o utcomes were measurement of alcohol and acetaldehyde in the blood after the alcohol inta ke. Blood levels of alcohol and acetaldehyde in the ALDH2 heterozygote group were signif icantly downregulated in the probiotic-supplemented group with no changes in hangover sc ore symptoms than the placebo group. No clinically significant changes were observed in s afety parameters. These results suggest that probiotic has a potential to downregulate the a lcohol and acetaldehyde concentrations, and their effects depend on the presence or absenc e of polymorphism on the ALDH2 gene.

Keywords: : Probiotics, *Lactobacillus, Bifidobacterium*, alcohol, acetaldehyde, ALDH2 gene

1. Introduction

Chronic alcohol consumption is one of the major causes of morbidity and mortality, ranging from simple steatosis to hepatocellular carcinoma [1]. Once ingested, alcohol is oxidized to toxic and carcinogenic acetaldehyde by alcohol dehydrogenase (ADH) and further oxidized to a non-toxic acetate by aldehyde dehydrogenase (ALDH) [2]. There are two major ALDH isoforms, cytosolic ALDH1 and mitochondrial ALDH2. Most Caucasians have two isozymes, while approximately 30%-50% of East Asians have ALDH2 deficiency that results from the inheritance of the mutant *ALDH2*2* allele [3]. Subjects with one or both alleles of *ALDH2*2* experience side effects, such as facial flushing, nausea, or vomiting after the alcohol consumption [4]. Probiotics are microorganisms that can change the gut lumen favoring an anti-inflammatory milieu, resulting in decreased pathogenic bacterial toxins and improved barrier integrity. *Lactobacilli* and *Bifidobacteria* are important members of the indigenous flora of the large intestine in humans and are also the best characterized and the most commercialized probiotics. The therapeutic potential of these probiotics on alcohol-induced liver diseases has been reported in animal and human studies [5-8]. Recently, Lu *et al*. [9] have shown that *Bacillus subtilis* co-expressing ADH and ALDH has a protective effect against the development of alcohol-induced liver damage in mice, suggesting that probiotics also play a key role in alcohol intoxication. However, no study has been conducted to evaluate whether probiotics influence alcohol metabolism in humans. Thus, in this investigation, a randomized, double-blind, placebo-controlled crossover study was performed to assess the capacity of *Lactobacilli* and *Bifidobacteria* to improve

alcohol metabolism. Also, their role in reducing hangover symptoms with respect to genetic variations of *ALDH2* was investigated.

2. Materials and Methods

2.1 Test supplements

Duolac ProAP4 constitutes four probiotics [*Lactobacillus gasseri* CBT LGA, *Lactobacillus casei* CBT LC5, *Bifidobacterium lactis* CBT BL3, and *Bifidobacterium breve* CBT BR3] and manufactured by Cell Biotech (Gimpo, Gyeonggi-do, Korea) [10]. It is double-coated and contained over 500,000,000 CFU/1.6 g of probiotics. Placebo was made of fructo-oligosaccharide and dextrose and had the same appearance, flavor, and weight as the Duolac products**.** According to the Ministry of Food and Drug Safety (MFDS)of Korea, intake of probiotics in healthy functional foods is $1*10^8$~10^{10} CFU per daily serving. In this study, the total number of probiotics ($1*10^9$ CFU/ml) was converted as per the intake standards, which was calculated to a total of 10^8~10^{10} CFU of probiotics per day, which is a MFDS notification type, was applied to the study. Previous pre-clinical studies show that serum alcohol and serum acetaldehyde concentrations were notably decreased in animals receiving Duolac ProAP4 administration [10]. Based on these results, the appropriate probiotic dose for subjects in the present study was 1,600 mg/day.

2.2. Subjects

This study was performed from 11th March to 26th October 2019 after receiving approval from the Institutional Review Board (IRB) of Jeonbuk National University Hospital (IRB No. JBNUH 2018-12-019). The entire study was conducted in accordance with the provisions of the Helsinki Declaration and the provisions of the Korean Good Clinical Practice (KGCP). The study was registered in the Clinical Research Information Service of Republic of Korea (Approval number: KCT0005361). All participants were instructed to take four whitening hard capsules per day (two capsules each after breakfast and dinner). Duolac ProAP4 and placebo capsules were packaged indistinguishably and labeled with a serial number. Participants were instructed to bring all the remaining supplements at each visit and were withdrawn from the study if the supplement consumption was < 80% of the prescribed dose. Alcohol challenge test was carried out on the 1st period (Day 15) and 2nd period (Day 58); after 30 min of standard meal intake. All participants consumed the day's supplements (Four capsules/day) with water.

The participants were recruited by advertising the investigation through various methods like brochures, posters, and JBNUH website. A total of 94 participants were eligible after screening tests such as questionnaires, physical examinations, genetic tests, and laboratory examinations. Participants were enrolled within four weeks after the screening test. Prior to the trial, informed consent was obtained from all the participants.

Inclusion criteria were as follows: (1) Male aged ≥19 and ≤65 years at the time of the screening test, (2) Body mass index (BMI) of 18 to 25 kg/m^2, (3) Healthy adults with post-drinking hangover experience and those who had fully understood the detailed description of the study and voluntarily agreed to participate. Exclusion criteria for the study were: (1) A person who is a homozygote type (ALDH2*2/*2) of the ALDH2 genotype, (2) A person who is hypersensitive or has a history of clinically significant hypersensitivity to drugs, alcohol, products, or other ingredients, (3) A person who has taken a drug that induces and inhibits drug metabolic enzymes, such as barbital drugs, within one month from the date of screening test, (4) A person who has taken drugs that affect the clinical results such as alcohol metabolism within one month from the screening test (drugs with a risk of gastrointestinal bleeding such as aspirin, antipyretic analgesics, anti-inflammatory analgesics, antibiotics, herbal medicines, oral steroids, hormones, etc.), (5) A person who has taken drugs, products, and health functional foods that are believed to affect the intestines, such as probiotics, *Lactobacillus* drinks (e.g., yogurt), and dairy products, within one month from the date of screening test, (6) A person who has taken drugs, products, and health functional foods that are believed to have an effect on the stomach and liver, such as milk thistle (silymarin) and licorice extract, within one month from the date of screening test, (7) A person who has taken drugs, products, and health functional foods that are deemed unsuitable for participation in the study by the person in charge of the study, such as hangover relief products. (8) A person who has consumed excessive alcohol within one week from the screening test date, (9) A person with severe acute or chronic cardiovascular diseases, metabolic diseases, liver and biliary diseases, pancreatic diseases, muscle diseases, neurological diseases, mental disorders, endocrine diseases, immune diseases, kidney diseases, malignant tumors, lung diseases, and other diseases requiring treatment, (10) A

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person who has or is undergoing treatment for a clinically significant gastrointestinal disease such as gastric or duodenal ulcer, (11) A person who has a history of a gastrointestinal disease such as Crohn's disease or gastrointestinal surgery (excluding simple appendectomy or herniotomy) that could affect the absorption of the study diet, (12) A person who has received antipsychotic drug within 2 months from the date of the screening test, (13) A person who has or is suspected of having a history of alcoholism or drug abuse, (14) A person who has participated in other studies within 3 months from the screening test date [except simple observational studies in which there was no intra-body administration of drugs or foods (injection, ingestion, insertion, etc.)], (15) A person who has donated whole blood within 2 months from the date of screening or donated apheresis within 2 weeks from the date of screening, (16) A person who has serum AST, ALT, or creatine kinase levels two times greater than the upper limit of the reference range or serum creatinine level over 2.0 mg/dL in diagnostic tests, and (17) A person who is deemed unfit for this study by the tester due to diagnostic test results or other reasons.

2.3. Genotyping

The ALDH2 gene was classified as wild type (ALDH2*1/*1), homozygote type (ALDH2*2/*2), and heterozygote type (ALDH2*2/*1) through single nucleotide polymorphism (SNP) r671 analysis. The variant ALDH2*2 type was caused by a single-point mutation (G-A) of Exon 12, which induces amino acid substitution from glutamine to lysine (E487K).

2.4. Study design

The study was designed as a randomized, double-blind, and placebo-controlled crossover trial **(Figure 1).** Participants who met the entry criteria and responded via a telephone screening interview were scheduled for a baseline visit. The evaluation included a physical test, electrocardiogram, and blood parameters. After obtaining the written informed consent, 54 participants were assigned to either group A (Duolac ProAP4 intake → washout → placebo intake) or group B (placebo intake → washout → Duolac ProAP4 intake). Alcohol challenge test was performed after an overnight fast on day 15 and day 58. The participants were asked to maintain their diet during the study period and avoid eating any related health functional foods or dietary supplements. Participants were also asked to report any adverse events or any changes in training, lifestyle, eating patterns, and pill compliance.

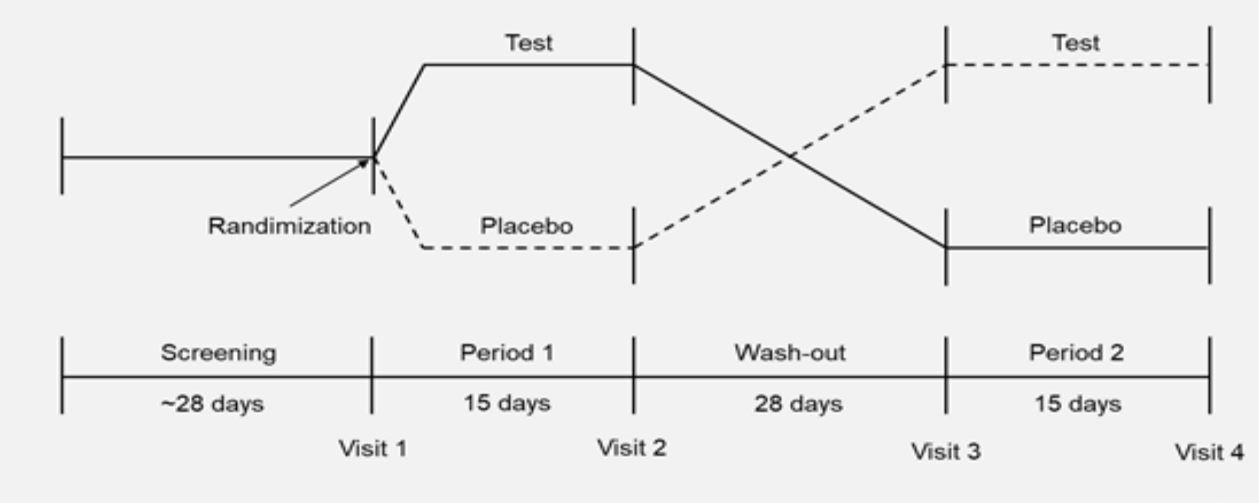

Figure 1. Scheme of the crossover design protocol

2.5. Alcohol challenge test

During the alcohol challenge test, participants had a meal (standard diet) with alcohol (40% v/v, Absolut Vodka, The Absolut Company AB, Stockholm, Sweden). Alcohol consumed with water at 1:1 ratio amounting to 0.8 g per kg body weight of the study participants and consumed within 30 min with a small amount of snack. Bodyweight was based on the measurements of the first and third visit. Blood levels of alcohol and acetaldehyde were measured at 0, 0.5, 1, 2, 4, and 6 hours after alcohol drinking.

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2.6. Outcome measurements

2.6.1. Primary outcomes

The primary outcomes were alcohol and acetaldehyde concentrations in the blood after the alcohol intake. Blood samples were obtained in anticoagulating tubes containing potassium-EDTA (BD Biosciences, San Jose, CA, USA) at baseline and at 0, 0.5, 1, 2, 4, and 6 hours after the alcohol administration. Blood alcohol concentration was detected by headspace gas chromatography with flame ionization detection (HS-GC-FID) [11]. A 100 uL of whole blood was diluted with 1000 uL of internal standard solution in each vial. The samples were determined on a HS-GC-FID system (6890GC-FID, Agilent Technologies, Santa Clara, CA, USA) with headspace autosampler (G1888A, Agilent Technologies, Santa Clara, CA, USA). The conditions of analysis were as follows: DB-624 column (30 m x 0.251 mm x 1.40 mm; Agilent Technologies, Wilmington, DE, USA); 0–30 min, oven temperature program (40°C for 3 min hold, 10mL/min up to 260°C, 5 min hold); headspace oven temperature, 80°C; sample heating time, 15 min.

Blood acetaldehyde concentration was detected by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring (MRM) [12]. Briefly, 1000 uL of whole blood was added in each vial containing mixture of 1 mL of saturated sodium nitrite and 100 uL of acetone for the internal standard. After adding 2, 4-dinitrophenylhydrazine (DNPH) cartridge, the mixtures were reacted for 24 hours in the dark condition. The samples were extracted with 1 mL acetonitrile and detected using 6410 Triple Quad LC-MS/MS (Agilent Technologies, Wilmington, USA). The analytical HPLC column was a reverse phase column (Shiseido CAPCELL, C18, 5um, 2.0mm* 10cm). The flow rate was 0.23 mL/min and the elution was done with a gradient of water and acetonitrile containing 0.1% formic acid. Fragmentor voltage and collision voltage were set at 100V and 10V. Detection of the ions was carried out with MRM by monitoring the transition pairs of m/z 225.1 → 208.3 (aldehyde-DNPH). Data acquisition was performed with the MassHunter Software (Version B.04.00, Agilent, Santa Clara, CA, USA). At the same time point, expiratory alcohol concentration was measured by Lion SD-400 Breath Alcohol Analyser (Lion Laboratories, Barry, Vale of Glamorgan, UK). Maximum plasma concentration (C_{max}), the time to reach it C_{max} (T_{max}), and the incremental area under the curve (iAUC) were calculated using the concentrations of alcohol and acetaldehyde in the blood, and trapezoidal method was used for calculating the iAUC used [13].

2.6.2. Secondary outcomes

The secondary outcomes were Alcohol Hangover Questionnaire (AHQ), liver function test, and blood glucose levels. AHQ was conducted within 8 h of alcohol consumption during the alcohol challenge test. AHQ consisted of 20 questions, including questions about thirst, sleepiness, headache, dizziness, vomiting, helplessness, abdominal pain, diarrhea, concentration difficulty, and sensitivity to irritation [14]. Liver enzymes tests (AST, ALT, ALP, and γ-GT) were measured at 0, 1, and 6 h after the alcohol consumption. Blood glucose levels were measured at 0 and 6 h after drinking alcohol.

2.7 Safety outcome measurements

At each visit, participants underwent electrocardiogram, laboratory tests (WBC, RBC, Hb, Hct, platelet, ALP, γ-GT, AST, ALT, total bilirubin, total protein, albumin, BUN, creatinine, creatine kinase, total cholesterol, triglyceride, glucose, and hs-CRP), and vital signs (systolic blood pressure, diastolic blood pressure, and pulse) for safety evaluation. WBC, RBC, Hb, Hct, and platelet were measured using automated hematology analyzer XE-5000TM (Sysmex, Kobe, Japan). ALP, γ-GT, AST, ALT, total bilirubin, total protein, albumin, BUN, creatinine, creatine kinase, total cholesterol, triglyceride, glucose, and hs-CRP were measured using the ADVIA® 2400 chemistry system (Siemens, Munich, Germany).

2.8 Evaluation of diet and physical activity

Three-day food and physical activity records were collected at each visit to evaluate the usual diet and physical activity patterns of the participants. Dietary intake was analyzed by the same dietitian using CAN-pro 4.0 software (The Korean Nutrition Society, Seoul, Korea), and physical activity was assessed using a metabolic equivalent task (MET) assessment using the global physical activity questionnaire (GPAQ) developed by the World Health Organization [15].

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2.9 Statistical analysis

Statistical analysis was performed using the SAS version 9.4 (SAS Institute, Charlotte, NC, USA). Analyses were performed according to intention-to-treat principles. For each variable, participants were grouped according to the sequence of intervention (Duolac ProAP4, then placebo or placebo, then Duolac ProAP4). The student's paired *t*-test was used for continuous measurements to assess differences between before and after the 15-day intervention period. Fixed effects included treatment group, treatment visit, and the interaction between treatment group and visit. Data are shown as the mean ± standard deviation (SD). A *p*-value less than 0.05 was considered statistically significant.

2.8 Sample size

Sample size was calculated to detect the blood acetaldehyde AUC changes 0.0079±0.0225 mg·h/dL between the Duolac ProAP4 and placebo groups. The sample size required to maintain 80% statistical power at a 5% significance level (two-tailed test) was calculated to be 40 persons per group. Therefore, a total of 54 people was required, assuming a dropout ratio of 25%.

3. Results

3.1. Demographic characteristics of participants

Among the 94 participants screened, 40 participants were excluded due to laboratory test results consistent with the exclusion criteria. The remaining 54 participants fulfilled the study criteria and included in the investigation. The supplement was consumed according to the order of intake of the assignment group, which was randomly assigned to either group A or group B (Group A: Duolac ProAP4, then placebo and Group B: placebo, then Duolac ProAP4). Also, the assigned group was stratified by the ALDH2 genotypes. According to the crossover design, participants received the opposite treatment after a 28-day washout period. During the study participation period, six people in group A and eight people in group B violated the human application test plan, 40 participants (21 in group A and 19 in group B) were able to finish the study **(Figure 2).** **Table 1** shows the general characteristics of the 54 participants. Baseline characteristics of age, height, weight, BMI, drinking, smoking, blood pressure, pulse, temperature, and thyroid-stimulating hormone (TSH) were not significantly different between the wild and heterozygote types.

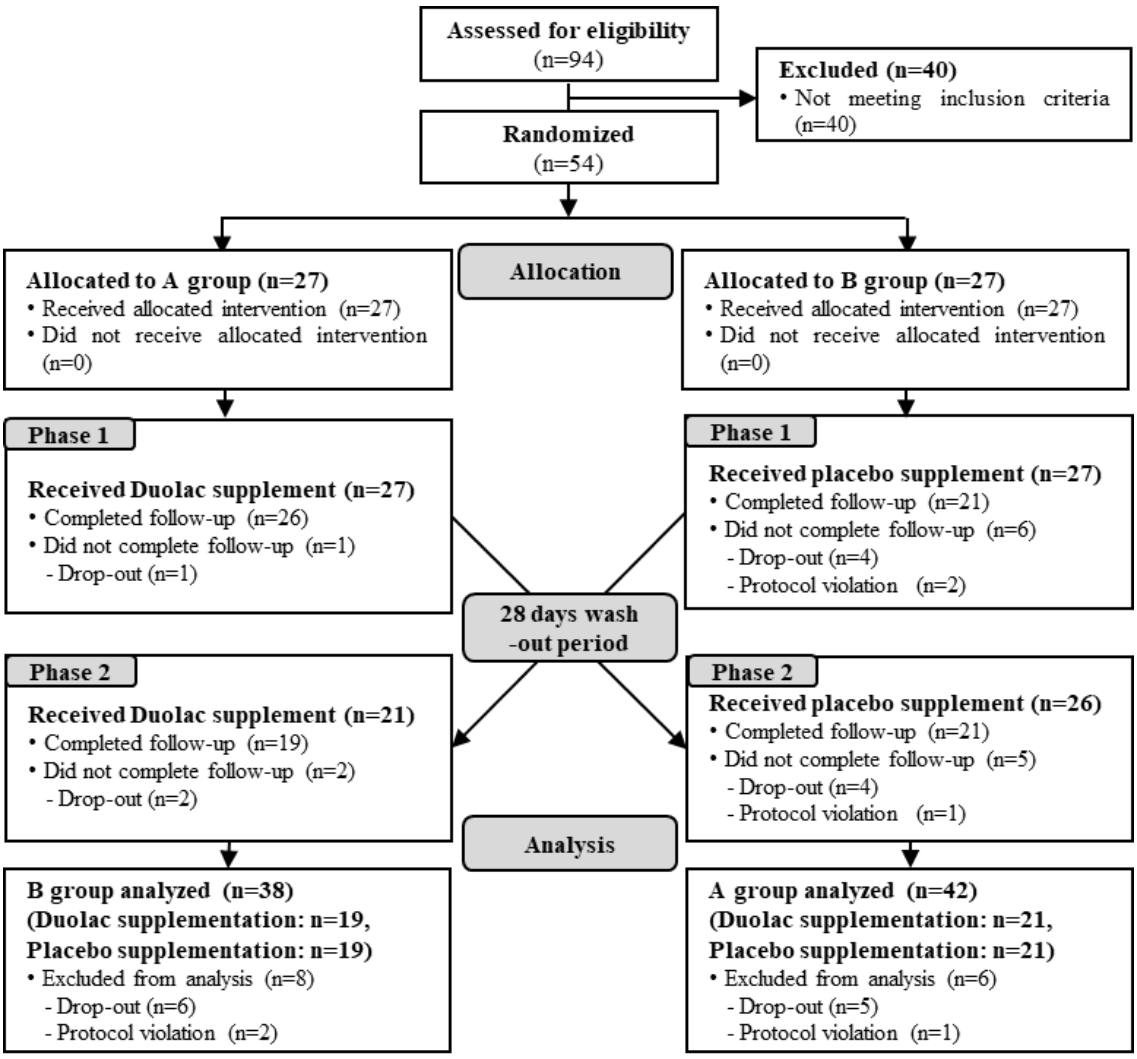

Figure 2. Flow diagram showing the selection & allocation of participants in the investigation

Table 1. Demographic characteristics of the study subjects

Variables		Wild type (ALDH2*1/*1, n=27)	Heterozygote (ALDH2*2/*1, n=27)	Total group (n=54)
Age (years)		25.26±2.61	24.89±2.97	25.07±2.77
Height (cm)		176.15±4.82	175.07±5.27	175.61±5.03
Weight (kg)		70.61±8.06	70.37±8.03	70.49±7.97
Body mass index (kg/m²)		22.77±2.14	22.93±1.84	22.85±1.98
Drinking (yes/no)	non-drinker (n, %)	0, 0	0, 0	0, 0
	past drinker (n, %)	0, 0	0, 0	0, 0
	drinker (n, %)	27, 100	27, 100	54, 100
Alcohol period (years)		6.00±1.96	5.81±2.32	5.91±2.13
Alcohol consumption (units/week)		7.38±2.41	4.13±2.22	5.75±2.82
	yes (n, %)	23, 85	25, 93	48, 89

Drinking within a week	no (n, %)	4, 15	2, 7	6, 11
Smoking	non-smoker (n, %)	17, 63	18, 67	35, 65
	past smoker (n, %)	0, 0	0, 0	0, 0
	Smoker (n, %)	10, 37	9, 33	19, 35
Smoking period (years)		6.30±2.71	3.67±2.40	5.05±2.84
Smoking consumption (units/week)		10.10±5.34	7.44±4.69	8.84±5.09
Smoking within a week	yes (n, %)	10, 100	8, 89	18, 95
	no (n, %)	0, 0	1, 11	1, 5
Systolic blood pressure (mmHg)		119.81±8.26	119.04±10.36	119.43±9.29
Diastolic blood pressure (mmHg)		71.70±8.88	70.48±8.17	71.09±8.47
Pulse (BPM)		80.48±9.93	72.56±7.71	76.52±9.67
Temperature		36.2±0.21	36.24±0.24	36.22±0.23
Thyroid stimulating hormone		1.84±1.32	1.73±0.65	1.79±1.03

Values are presented as mean±SD or frequency (%).

3.2 Diet intake and physical activity

Significant differences in dietary intakes (calories, carbohydrates, protein, fat, and fiber) or physical activity (MET) were not confirmed between the groups during the intervention period (data not shown).

3.3 Efficacy evaluation

3.3.1. Primary outcome

Table 2 shows the variation in blood acetaldehyde concentration after 15 days of Duolac ProAP4 supplementation. In the heterozygote group, Duolac ProAP4 supplementation clearly accelerated alcohol metabolism as acetaldehyde concentrations at 0.5, 1, and 6 h after alcohol consumption, and C_{max}, and iAUC were significantly lower in Duolac ProAP4 supplemented participants compared with those of placebo group ($p<0.05$). However, these effects were not observed in wild-type participants. Alcohol concentrations were higher in heterozygote group regardless of Duolac ProAP4 supplementation compared to those in wild-type group. To reiterate, Duolac ProAP4 supplementation significantly decreased the alcohol concentration in the heterozygote group compared to the placebo group **(Table 3).**

Table 2. Variation in blood acetaldehyde concentration flowing alcohol challenge test after 15 days of supplementation

		Wild type (ALDH2*1/*1)			Heterozygote (ALDH2*2/*1)			Total group		
		Duolac group (n=19)	Placebo group (n=19)	*p*-value[1)]	Duolac group (n=21)	Placebo group (n=21)	*p*-value[1)]	Duolac group (n=40)	Placebo group (n=40)	*p*-value[1)]
Blood acetaldehyde level (mg/dl)	0 h	0.0000 ±0.0000	0.0008 ±0.0021	0.117	0.0001 ±0.0004	0.0002 ±0.0011	0.553	0.0004 ±0.0002	0.0005 ±0.0002	0.094
	0.5 h	0.0072 ±0.0003	0.0045 ±0.0063	0.660	0.1128 ±0.0588	0.1496 ±0.0853	**0.018**	0.0626 ±0.0701	0.0807 ±0.0956	**0.040**
	1 h	0.0043 ±0.0104	0.0054 ±0.0101	0.773	0.1075 ±0.0634	0.1465 ±0.0919	**0.005**	0.0585 ±0.0695	0.0795 ±0.0974	**0.006**
	2 h	0.0020 ±0.0041	0.0021 ±0.0038	0.941	0.0497 ±0.0417	0.0651 ±0.0521	0.130	0.0270 ±0.0385	0.0352 ±0.0492	0.129
	4 h	0.0000 ±0.0000	0.0002 ±0.0006	0.181	0.0191 ±0.0255	0.0278 ±0.0370	0.197	0.0100 ±0.0207	0.0147 ±0.0300	0.184
	6 h	0.0000 ±0.0000	0.0001 ±0.0005	0.331	0.0049 ±0.0057	0.0098 ±0.0099	**0.020**	0.0026 ±0.0048	0.0052 ±0.0086	**0.019**
C_{max} (mg/dl)		0.0079 ±0.0252	0.0065 ±0.0100	0.829	0.1214 ±0.0645	0.1702 ±0.0961	**0.002**	0.0675 ±0.0756	0.0924 ±0.1078	**0.007**
T_{max}		0.68±0.38	0.63±0.23		0.76±0.26	0.71±0.25		0.73±0.32	0.68±0.24	
Median (min-max)		0.50 (0.50-2.00)	0.50 (0.50-1.00)	0.542	1.00 (0.50-1.00)	0.50 (0.50-1.00)	0.329	0.50 (0.50-2.00)	0.50 (0.50-1.00)	0.291
iAUC (mg · hr/dl)		0.0098 ±0.0244	0.0079 ±0.0140	0.774	0.2541 ±0.1732	0.3465 ±0.2362	**0.022**	0.1380 ±0.1759	0.1856 ±0.2409	**0.029**

Values are presented as mean ± SD.

Abbreviation: C_{max}, maximum plasma concentration; T_{max}, time to reach C_{max}; iAUC, incremental area under the curve

[1)] Analyzed using paired *t*-test (compared between groups)

Table 3. Variation in blood alcohol concentration flowing alcohol challenge test after 15 days of supplementation

		Wild type (ALDH2*1/*1)			Heterozygote (ALDH2*2/*1)			Total group		
		Duolac group (n=19)	Placebo group (n=19)	*p*-value[1)]	Duolac group (n=21)	Placebo group (n=21)	*p*-value[1)]	Duolac group (n=40)	Placebo group (n=40)	*p*-value[1)]
Blood alcohol level (mg/dl)	0 h	0.00 ±0.00	0.00 ±0.00	-	0.00 ±0.00	0.00 ±0.00	-	0.00 ±0.00	0.00 ±0.00	-
	0.5 h	62.71 ±29.85	66.27 ±27.54	0.558	81.34 ±31.55	77.28 ±29.39	0.425	72.49 ±31.79	72.05 ±28.70	0.909

	1 h	85.54 ±24.08	90.35 ±23.44	0.348	90.80 ±24.72	92.38 ±15.98	0.750	88.30 ±24.25	91.42 ±19.64	0.374
	2 h	82.37 ±10.05	81.51 ±16.79	0.769	74.57 ±24.19	79.40 ±20.01	0.123	78.27 ±19.03	80.40 ±18.34	0.320
	4 h	49.53 ±9.60	51.05 ±14.72	0.511	57.16 ±22.64	62.46 ±20.23	0.159	53.54 ±17.90	57.04 ±18.52	0.116
	6 h	11.98 ±7.79	16.69 ±9.0	**0.009**	25.03 ±13.44	31.99 ±14.94	**0.039**	18.83 ±12.81	24.73 ±14.56	**0.002**
C_{max} (mg/dl)		92.39 ±18.0	91.98 ±21.16	0.909	94.35 ±28.50	96.48 ±17.89	0.673	93.42 ±23.82	94.34 ±19.39	0.763
T_{max}		1.37±0.57	1.18±0.45		0.95±0.31	1.19±0.83		1.15±0.50	1.19±0.67	
Median		1.00	1.00	0.185	1.00	1.00	0.180	1.00	1.00	0.744
(min-max)		(0.50-2.00)	(0.50-2.00)		(0.50-2.00)	(0.50-4.00)		(0.50-2.00)	(0.50-2.00)	
iAUC (mg ・ hr/dl)		330.11 ±56.49	341.95 ±81.03	0.361	359.97 ±118.97	383.95 ±93.41	0.127	345.79 ±94.65	363.40 ±89.20	0.072

Values are presented as mean ± SD.

Abbreviation: C_{max}, maximum plasma concentration; T_{max}, time to reach C_{max}; iAUC, incremental area under the curve

[1)] Analyzed using paired *t*-test (compared between groups)

3.3.2. Secondary outcomes

The alcohol challenge test after ingestion of the test products in this study revealed a notable difference between the two groups as the ALP levels after and before alcohol consumption in the Duolac ProAP4 group decreased compared to the placebo group (p=0.001). The analysis of the hetero-type group, the liver enzymes of AST (1 h), ALT (1 h), and ALP (6 h) in the Duolac ProAP4 group were significantly decreased compared to the placebo group ($p<0.05$). However, in the wild-type, there was no significant difference in liver enzymes between Duolac ProAP4 and placebo groups **(Table 4).**

AHQ of hangover symptom index was measured within 8 h of the alcohol consumption **(Table 5).** The sum of all the items in each AHQ, the sum of 13 major symptoms of hangover [16], and the sum of score of 7 items [17] were compared. In contrast to the changes of alcohol and acetaldehyde concentrations, there were no significant difference between the two groups in the total score, score of 13 major hangover symptoms, and score of 7 items. Heart palpitations (Q15) and elated mood (Q17) were significantly worsened in heterozygote type.

Table 4. Variation in serum liver enzymes level flowing alcohol challenge test after 15 days of supplementation

		Wild type (ALDH2*1/*1)					Heterozygote (ALDH2*2/*1)					Total group				
Liver enzymes (standard range)	Time	Duolac group (n=19)	Diff	Placebo group (n=19)	Diff	*p*-value[1)]	Duolac group (n=21)	Diff	Placebo group (n=21)	Diff	*p*-value[1)]	Duolac group (n=40)	Diff	Placebo group (n=40)	Diff	*p*-value[1)]
AST (12~33 IU/L)	0 h	22.79 ±4.43	-	23.47 ±8.64	-	0.752	21.14 ±4.52	-	20.57 ±4.93	-	0.574	21.93 ±4.50	-	21.95 ±7.01	-	0.983
	1 h	22.16 ±5.00	-0.63 ±2.09	22.53 ±8.12	-0.95 ±1.99	0.672[2)]	21.00 ±3.97	-0.14 ±2.22	21.90 ±5.46	1.33 ±2.03	**0.032**[2)]	21.55 ±4.47	-0.38 ±2.14	22.20 ±6.77	0.25 ±2.30	0.217[2)]
	6 h	23.53 ±5.73	0.74 ±2.62	23.11 ±7.52	-0.37 ±2.79	0.241[2)]	21.48 ±4.11	0.33 ±1.91	21.52 ±4.73	0.95 ±1.99	0.374[2)]	22.45 ±4.99	0.53 ±2.25	22.28 ±6.18	0.33 ±2.46	0.728[2)]
ALT (5~35 IU/L)	0 h	25.42 ±10.17	-	25.84 ±14.66	-	0.903	24.00 ±9.59	-	23.05 ±10.13	-	0.598	24.68 ±9.77	-	24.38 ±12.4	-	0.871
	1 h	24.47 ±10.40	-0.95 ±2.76	25.16 ±14.65	-0.68 ±3.00	0.810[2)]	22.00 ±9.64	-2.00 ±3.00	23.24 ±9.32	0.19 ±3.40	**0.029**[2)]	23.18 ±9.96	-1.50 ±2.90	24.15 ±12.02	-0.23 ±3.21	0.082[2)]
	6 h	24.74 ±10.44	-0.68 ±3.54	24.95 ±14.19	-0.89 ±2.81	0.864[2)]	22.24 ±9.97	-1.76 ±2.96	22.10 ±9.90	-0.95 ±2.89	0.402[2)]	23.43 ±10.14	-1.25 ±3.26	23.45 ±12.06	-0.93 ±2.81	0.669[2)]
ALP (45~129 IU/L)	0 h	62.26 ±11.58	-	59.53 ±11.30	-	0.069	64.57 ±12.27	-	59.62 ±12.74	-	0.010	63.48 ±14.70	-	59.58 ±11.92	-	0.001
	1 h	62.74 ±11.11	0.47 ±3.13	60.89 ±11.44	1.37 ±2.61	0.371[2)]	66.86 ±17.24	2.29 ±3.65	62.48 ±13.28	2.86 ±2.71	0.505[2)]	64.90 ±14.62	1.43 ±3.49	61.73 ±12.31	2.15 ±2.73	0.259[2)]
	6 h	61.84 ±11.56	-0.42 ±3.19	60.58 ±11.21	1.05 ±2.46	0.106[2)]	64.05 ±16.84	-0.52 ±2.79	61.86 ±13.76	2.24 ±2.55	**0.003**[2)]	63.00 ±14.44	-0.48 ±2.94	61.25 ±12.47	1.68 ±2.65	**0.001**[2)]
	0 h	25.05	-	24.63	-	0.814	17.48	-	18.05	-	0.616	21.08	-	21.18	-	0.922

		±13.36		±14.01			±6.31		±5.55			±10.84		±10.84		
γ-GT (12~73 IU/L)	1 h	23.16 ±12.46	-1.89 ±2.51	23.37 ±13.44	-1.26 ±2.83	0.448[2)]	15.33 ±6.19	-2.14 ±1.98	15.86 ±4.98	-2.19 ±3.37	0.957[2)]	19.05 ±10.35	-2.03 ±2.22	19.43 ±10.51	-1.75 ±3.12	0.645[2)]
	6 h	23.26 ±11.74	-1.79 ±3.34	23.58 ±13.64	-1.05 ±2.30	0.419[2)]	16.67 ±5.13	-0.81 ±2.34	16.62 ±5.40	-1.43 ±3.30	0.508[2)]	19.80 ±9.39	-1.28 ±2.86	19.93 ±10.64	-1.25 ±2.84	0.969[2)]

Values are presented as mean ± SD.

1) Analyzed using paired *t*-test (compared between groups).

2) Analyzed using paired *t*-test (difference between change values).

Table 5. Score of alcohol hangover questionnaire after 15 days of supplementation

	Wild type (ALDH2*1/*1)			Heterozygote (ALDH2*2/*1)			Total group		
Hangover symptom index	Duolac group (n=19)	Placebo group (n=19)	*p*-value[1)]	Duolac group (n=21)	Placebo group (n=21)	*p*-value[1)]	Duolac group (n=40)	Placebo group (n=40)	*p*-value[1)]
Q1	2.05±1.03	2.16±1.07	0.578	2.67±1.11	2.76±0.83	0.680	2.38±1.10	2.48±0.99	0.500
Q2	2.84±1.12	2.89±1.29	0.841	3.62±1.16	3.71±0.85	0.693	3.25±1.19	3.33±1.14	0.667
Q3	2.63±1.34	2.68±1.29	0.790	2.76±0.94	2.86±1.15	0.715	2.70±1.14	2.78±1.21	0.645
Q4	1.84±1.07	2.42±1.07	0.053	2.57±1.03	2.52±1.12	0.853	2.23±1.10	2.48±1.09	0.201
Q5	1.37±0.83	1.47±0.70	0.630	1.81±0.98	1.62±0.80	0.258	1.60±0.93	1.55±0.75	0.711
Q6	1.68±0.58	1.89±0.88	0.360	2.14±1.20	2.38±1.07	0.234	1.93±0.97	2.15±1.00	0.130
Q7	1.05±0.23	1.05±0.23	>.999	1.19±0.40	1.29±0.56	0.493	1.13±0.33	1.18±0.45	0.534
Q8	1.05±0.23	1.32±0.67	0.135	1.05±0.22	1.14±0.48	0.428	1.05±0.22	1.23±0.58	0.090
Q9	1.74±0.87	1.95±0.71	0.331	2.00±1.22	2.24±1.14	0.366	1.88±1.07	2.10±0.96	0.183
Q10	1.26±0.45	1.32±0.48	0.667	1.33±0.66	1.62±0.92	0.162	1.30±0.56	1.48±0.75	0.147
Q11	1.32±0.82	1.16±0.50	0.380	1.05±0.22	1.24±0.54	0.104	1.18±0.59	1.20±0.52	0.812
Q12	1.42±0.61	1.16±0.37	0.056	1.48±0.98	1.33±0.66	0.576	1.45±0.81	1.25±0.54	0.173
Q13	1.16±0.69	1.00±0.00	0.331	1.05±0.22	1.10±0.30	0.576	1.10±0.50	1.05±0.22	0.570
Q14	1.16±0.37	1.53±1.12	0.185	1.05±0.22	1.14±0.36	0.329	1.10±0.30	1.33±0.83	0.107
Q15	1.32±0.58	1.11±0.32	0.163	3.24±1.04	2.62±1.36	**0.024**	2.33±1.29	1.90±1.26	**0.008**
Q16	1.26±0.56	1.32±0.58	0.749	1.57±0.81	1.57±0.98	>.999	1.43±0.71	1.45±0.81	0.838
Q17	2.00±1.00	1.79±0.92	0.494	2.57±1.21	2.05±1.12	**0.018**	2.30±1.14	1.93±1.02	**0.042**
Q18	1.63±0.90	1.58±0.84	0.772	4.00±1.14	3.81±1.08	0.446	2.88±1.57	2.75±1.48	0.418
Q19	1.79±0.85	1.63±0.83	0.604	3.52±1.29	3.29±1.15	0.309	2.70±1.40	2.50±1.30	0.282

Q20	1.11±0.32	1.00±0.00	0.163	2.57±1.29	2.33±1.06	0.204	1.88±1.20	1.70±1.02	0.090
Symptom index (20)*	31.68±8.09	32.42±6.99	0.752	43.24±10.14	42.62±10.46	0.734	37.75±10.82	37.78±10.26	0.986
Symptom index (13)#	21.26±5.09	22.84±5.25	0.305	24.71±6.01	25.71±6.22	0.395	23.08±5.79	24.35±5.89	0.174
Symptom index (7)†	12.21±3.14	12.89±3.49	0.407	14.76±4.35	15.52±3.83	0.339	13.55±3.99	14.28±3.86	0.197

Values are presented as mean ± SD.

1) Analyzed using paired *t*-test (compared between groups)

(Q1) excessive thirst, (Q2) sleepiness, (Q3) headache, (Q4) dizziness, (Q5) vomiting, a sense of helplessness, (Q6) lack of energy, (Q7) abdominal pain, (Q8) diarrhea, (Q9) concentration difficulty, (Q10) more sensitive to irritation than usual (light and sound), (Q11) sleep difficulty, (Q12) sweat more than usual (sticky sweat), (Q13) melancholy, (Q14) memory disconnection, (Q15) heart palpitations, (Q16) Zone (nausea = feels like vomiting is urgent), (Q17) elated mood, (Q18) blush of the face, (Q19) body warms up, (Q20) shortness of breath

* Excessive thirst, sleepiness, headache, dizziness, vomiting, a sense of helplessness, lack of energy, abdominal pain, diarrhea, concentration difficulty, more sensitive to irritation than usual (light and sound), sleep difficulty, sweat more than usual (sticky sweat), melancholy, memory disconnection, heart palpitations

Zone (nausea = feels like vomiting is urgent), elated mood, blush of the face, body warms up, shortness of breath

Excessive thirst, sleepiness, headache, dizziness, vomiting, a sense of helplessness, abdominal pain, diarrhea, concentration difficulty, more sensitive to irritation than usual (light and sound), sweat more than usual (sticky sweat), melancholy, memory disconnection

† Excessive thirst, sleepiness, headache, dizziness, a sense of helplessness, concentration difficulty, heart palpitations, zone (nausea = feels like vomiting is urgent)

3.4 Safety and adverse events

No serious adverse events were reported during the study period. The laboratory tests, electrocardiogram, and vital signs were in the normal range (data not shown). Thus, no participants withdrew because of adverse events.

4. Discussion

Previously, Cell Biotech Co Ltd has screened 19 CBT probiotic species of *Lactobacillus* and *Bifidobacterium* to choose the best combination of probiotic strains for alcohol detoxification. In that investigation, they found that *Lactobacillus gasseri* CBT LGA1, *Lactobacillus casei* CBT LC5, *Bifidobacterium lactis*, CBT BL3 and *Bifidobacterium breve* CBT BR3 were highly effective in alcohol metabolism [10]. Specifically, *Lactobacillus gasseri* CBT LGA1 and *Bifidobacterium lactis* CBT BL3 demonstrated a high capacity for ethanol metabolism, while *Lactobacillus casei* CBT LC5 and *Bifidobacterium breve* CBT BR3 accelerated acetaldehyde metabolism. Further, the mixture of these four probiotics (Duolac ProAP4) was observed to benefit acute alcohol toxicity in rats [10]. Here, we evaluated the effect of Duolac ProAP4 on alcohol detoxification in humans. Consistent with the animal study, this randomized placebo-controlled crossover study demonstrates that Duolac ProAP4 supplementation results in lower blood concentrations of alcohol and acetaldehyde in the

heterozygote (ALDH2*2/*1) subjects, but not in wild-type (ALDH2*1/*1) subjects. However, there were no marked improvements in hangover parameters between the test and placebo groups. These observations distinctly suggest that Duolac ProAP4 supplementation is an effective way to maintain lower alcohol and acetaldehyde concentrations in humans.

Previously, Cell Biotech Co Ltd and other groups have shown that *Lactobacillus* and *Bifidobacterium* species encode ADH and ALDH [18-21]. In this study, Duolac ProAP4 supplementation significantly decreased plasma concentrations of acetaldehyde 1 h after the alcohol ingestion compared with those of placebo group (0.1075±0.0634 mg/dL in Duolac ProAP4 group vs. 0.1465±0.0919 mg/dL in placebo group, p=0.005) cemented the previously observed notion. However, Duolac ProAP4 supplementation did not affect alcohol levels 30 min after the alcohol ingestion (81.34±31.55 mg/dL in Duolac ProAP4 group vs. 77.28±29.39 mg/dL in placebo group, p=0.425). These results indicate that ProAP4 does not affect the alcohol breakdown and its absorption in the stomach instantly, but it accelerates acetaldehyde oxidation into acetate in the intestine. Interestingly, Duolac ProAP4 supplementation significantly decreased the blood concentrations of acetaldehyde 6 h after the alcohol ingestion compared with those of placebo group (0.0026±0.0048 mg/dL in Duolac ProAP4 group vs. 0.0052±0.0086 mg/dL in placebo group, p=0.019). These results suggest that Duolac ProAP4 may also increase acetaldehyde metabolism in the liver. Previously, probiotic products containing Lactobacillus and Bifidobacterium actively promoted alcohol metabolism where it rapidly decompose alcohol and metabolizes it to acetaldehyde, a harmful compound to the human body[22]. Previous studies have reported on the possibility of detoxification. In line with these studies, Cell Biotech Co Ltd and others have shown that probiotics supplementation has positive effects, alleviating acute alcoholic liver injury [5-8,18,23].

Heterozygote subjects taking Duolac ProAP4 showed an evident suppression in alcohol and acetaldehyde concentrations over time. However, those changes were not found in the wild-type subjects. These observations are unexpected, and it is difficult to explain these findings from the viewpoint of Duolac ProAP4's ALDH enzyme activity. One possible speculation is the difference in the gut microbiota community between the two groups. It is well documented that subjects with a single nucleotide polymorphism on ALDH2 gene tended to avoid excess alcohol drinking because of unpleasant hangover symptoms secondary to the failure of acetaldehyde metabolism [3,4]. Differences in alcohol ingestion potentially affect the composition of bowel flora. Evidently, alcoholics demonstrated to have reduced numbers of *Lactobacilli*, *Bifidobacteria*, and *Enterococci*, while there is an increase in the population of *E. coli* [8]. Similarly, animal studies have also reported a strong association between alcohol consumption and bowel flora composition [24,25]. Indeed, when we carefully compared the alcohol drinking history, we found that although there was no statistical significance, heterozygote subjects took less amount of alcohol compared to the wild-type subjects. Meanwhile, our study showed that Duolac ProAP4 supplementation substantially reduced blood acetaldehyde levels but did not relieve hangover symptom scores in heterozygote and wild-type subjects.. These findings are contrary to our expectation that acetaldehyde is the main contributor to the development of hangover symptoms. However, other factors like inflammatory cytokines, fluid imbalance, gender, ethnicity, genetic background, and nutritional status are associated with the frequency and severity of hangover symptoms along with blood acetaldehyde concentrations [26]. Thus, future studies are certainly needed to analyze the aforementioned parameters.

5. Conclusions

The present findings suggest that the mixture of four probiotics (Duolac ProAP4) is practically handy in the management of alcohol metabolism in the ALDH2*2/*1 subjects. Also, study warrants a large-scale clinical study to test if Duolac ProAP4 could be used to treat individuals with hangover symptoms after alcohol drinking.

Additional files: Additional file 1- CONSORT 2010 checklist*.

Author contributions: SJJ and JHH wrote the manuscript with input from all the authors**.** SWC, SJJ, BHP, SOL, JHH, YJC, and EOP conceived and designed the study. SJJ, JHH, YJC, SOL, BHP, and SWC conducted the investigation. JHH and EOP performed the statistical analysis. SWC, PBH, and SJJ supervised and participated in data interpretation. SWC, SJJ, MJC, SHL, TJL, and YHH involved in methodology. All the authors participated in the analyses of the biochemical data, interpretation of the data, and review of the paper. All the authors read and approved the final manuscript.

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