ISSN (electrónico): 1699-5198 - ISSN (papel): 0212-1611 - CODEN NUHOEQ S.V.R. 318

ARÁN

Nutrición Hospitalaria

Trabajo Original

Obesidad y síndrome metabólico

A randomized double-blind controlled clinical trial demonstrating efficacy of different probiotic strains on serum lipids and glycemic biomarker

Un ensayo clínico controlado aleatorizado, doble ciego, que demuestra la eficacia de diferentes cepas de probióticos en los lípidos séricos y el biomarcador glucémico

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Abstract

Background: the aim of this randomized placebo-controlled study was to investigate the effect of probiotics mainly on plasma lipids, homocysteine levels, glycemic biomarkers and inflammatory marker in people with hyperlipidemia, compared to a placebo.

Methods: a randomized, double-blind placebo-controlled study was completed with a total of 51 men and women who have diagnosed with hyperlipidemia. The three study interventions were: 1) probiotic group I asked to take once a day 1 x 10^6 colony forming unit (CFU) *Lactobacillus rhamnosus GG* microorganism ($n = 18$) capsule; 2) probiotic group II asked to take once a day of a combined *Lactobacillus acidophilus* 1 x 10^9 CFU and *Bifidobacterium animalis* subsp.*lactis* 1 x 10^9 CFU probiotic capsule ($n = 17$); and 3) placebo group: emptied capsule ($n = 16$), plasma lipids, homocysteine, and glycemic biomarkers were were performed at baseline and week 8. Also, hs-CRP levels was assessed as inflammatory parameter.

Results: compared to baseline there was a significant decrease in triglyceride and total cholesterol levels of the both intervention groups compared to the placebo group. Regarding the glycemic biomarkers. both intervention groups significantly alter the HOMA-IR values compared to the placebo group ($p < 0.05$). When homocysteine values were evaluated. a statistically significant decrease was observed only in the group using the combined strain ($p < 0.05$). Results demonstrated that regular and strain-specific use of probiotics have effective and favorable consequences on plasma lipids and glycemic biomarkers.

Conclusion: probiotics containing *Lactobacillus* or *Bifidobacterium* could be effective in hypercholesterolemic patients, reducing serum lipids as well as homocysteine and glycaemia.

Keywords:

Serum lipids.
Cardiovascular disease.
Cholesterol. Hyperlipidemia.
Probiotic.

Received: 11/04/2023 • Accepted: 23/05/2024

Ethical approval and consent to participate: before participating in the study, all participants were asked to sign a written consent form declaring that they wanted to voluntarily participate in the study, after being informed about the study.

Authors' contributions: Okburan G, Bas M and Ogmen S equally contributed to the conception and design of the research; Okburan G contributed to the design of the research; Okburan G contributed to the acquisition and analysis of the data; Okburan G and Ogmen S contributed to the interpretation of the data; Okburan G drafted the manuscript. Okburan G wrote the article with the supervision of Bas M. All authors critically revised the the manuscript, agree to be fully accountable for ensuring the integrity and accuracy of the work, and read and approved the final manuscript.

Acknowledgements: we would likte to thank all the individuals who participated in the study.

Conflict of interest: the authors declare no conflict of interest.

Artificial intelligence: the authors declare not to have used artificial intelligence (AI) or any AI-assisted technologies in the elaboration of the article.

Okburan G, Baş M, Ogmen S. A randomized double-blind controlled clinical trial demonstrating efficacy of different probiotic strains on serum lipids and glycemic biomarker. Nutr Hosp 2024;41(4):793-803
DOI: http://dx.doi.org/10.20960/nh.04724

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Resumen

Objetivo: el objetivo de este estudio aleatorizado controlado con placebo fue investigar el efecto de los probióticos principalmente en los lípidos plasmáticos, los niveles de homocisteína, los biomarcadores glucémicos y el marcador inflamatorio en personas con hiperlipidemia, en comparación con un placebo.

Métodos: se realizó un estudio doble ciego aleatoria controlado con placebo con un total de 51 hombres y mujeres a quienes se les había diagnosticado hiperlipidemia. Las tres intervenciones del estudio fueron: 1) un grupo probiótico que tomaban una vez al día 1 x 10^6 cápsulas de unidades formadoras de colonias (UFC) del microorganismo *Lactobacillus rhamnosus GG* ($n = 18$); 2) un grupo probiótico II que tomaba una vez al día una cápsula probiótica combinada de *Lactobacillus acidophilus* 1 x 10^9 CFU y *Bifidobacterium animalis* subsp.*lactis* 1 x 10^9 CFU ($n = 17$); y 3) un grupo placebo: cápsula vacía ($n = 16$), lípidos plasmáticos. Se realizaron biomarcadores de homocisteína y glucémico al inicio y también en la semana 8. Los niveles de hs-CRP se evaluaron como parámetro inflamatorio.

Resultados: en comparación con el valor inicial, hubo una disminución significativa en los niveles de triglicéridos y colesterol total de ambos grupos de intervención en comparación con los del grupo de placebo. En cuanto a los biomarcadores glucémicos, ambos grupos de intervención alteran significativamente los valores de HOMA-IR en comparación con el grupo placebo ($p < 0,05$). Cuando se evaluaron los valores de homocisteína, se observó una disminución estadísticamente significativa solo en el grupo que utilizó la cepa combinada ($p < 0,05$). Los resultados demostraron que el uso regular y específico de cepas de probióticos tiene consecuencias favorables sobre los lípidos plasmáticos y los biomarcadores glucémicos.

Conclusión: los probióticos que contienen *Lactobacillus* o *Bifidobacterium* podrían ser eficaces en pacientes hipercolesterolémicos, reduciendo los lípidos séricos, así como la homocisteína y la glucemia.

Palabras clave:

Lípidos en sangre.
Enfermedad cardiovascular.
Colesterol. Hiperlipidemia.
Probiótico.

INTRODUCTION

Hypercholesterolemia is a major risk factor for coronary artery disease and myocardial infarction (1,2). Regarding the lifestyle intervetion, dietary management, behavioural modifications and exercise are advised in order to lower the plasma cholesterol level (3,4). However, in some cases, these precautions may not be efficient and sufficient to manage the plasma lipids. Despite changes in lifestyle, in individuals with resistant high serum lipids they may require additional pharmacological treatment yet those treatment may associated with some adverse effects (5). In this context, for the treatment of high cholesterol level, alternative therapies or options are being investigated. One of the non-pharmacological approach for improving plasma lipids is the use of safe and strain-specific probiotics (6). Probiotics are defined as "live microorganisms which when administered in adequate amount confer health benefits to the host" (FAO/WHO, 2002) (7). Since the scientific evidence which is pointing the beneficial effects of probiotic on human health is becoming increasingly popular, there is an intense focus on probiotic bacteria and their health benefits (8). Some of the *in vitro* and animal studies indicated that strain-specific probiotics have cholesterol lowering effect via different mechanisms. Possible mechanisms than can be attributed to the hypocholesterolemic effect of probiotics include; deconjugation of bile acids by bile salt hydrolase (9), production of short chainf fatty acids (SCFAs) (10), and assimilation of cholesterol and fatty acids into the cell surface of the organism which makes cholesterol less available for absorption into the circulation (11). Regarding the hypocholesterolemic effect of probiotics, in contrast to *in vitro* and animal studies (12,13), human studies are not as consistent as *in vitro* studies (14-16). The reason for inconsistent results may attributed to the different type of strains that have been used, doses of probiotics, delivery matrix, study duration, and study population. The present study is one of the scarce studies that evaluated the effects of different probiotic strains on serum lipids, glycemic parameters, CRP and homocysteine. This double blind, placebo controlled study was conducted to demonstrate the efficacy of different probiotic strain use on serum lipids and glycemic biomarkers in healthy adults with hypercholesterolemia. In addition, it was evaluated whether different strains of probiotics differed on serum lipids and which could cause a more effective reduction.

EXPERIMENTAL METHODS

STUDY POPULATION AND DESIGN

A double-blind, placebo-controlled, parallel design randomised clinical trial was conducted in the Internal Medicine Department of Famagusta State Hospital to determine the effects of different probiotics on hyperlipidemia for 8 weeks. Patients diagnosed with hypercholesterolemia (defined as a total cholesterol ≥ 200 mg/dL) during a routine check-up in the Internal Medicine Department of Famagusta State Hospital, were eligible to participate in the study. Participants age ranges were between 30-64 (18 males, 33 females). Participants who met the the inclusion criteria were randomly assigned to either *Lactobacillus* group or *Lactobacillus* plus *Bifidobacterium* group or placebo group. Five visits were conducted; one prior to the study to screen and collect baseline data and to record food consumption and physical activity, anthropometry, and body composition measurements; the remaining interviews were every 15 days during the study period. Nutrient composition was determined with BEBIS Nutrition Data Base Software, physical activity was assessed on the same day with dietary records by average daily physical activity and expressed as physical activity level (PAL). Blood samples of the participants were collected twice: at the begining and at the end of the study. Participants in all groups were instructed to maintain their normal daily activity and nutritional habits during the study period. In order to remind the participants about the study capsules, daily messages was sent via Whatsapp. Prior to the contribution, each volunteer provided written informed consent.

ELIGIBILITY CRITERIA

All individuals who met the inclusion and exclusion criteria were invited to participate in the study. The inclusion criteria were: had a repeated total cholesterol level ≥ 200 mg/dL prior to allocate to the study group and to declined conventional lipid lowering medical treatment. Those with any chronic conditions other than hypercholesterolaemia, inherited lipid metabolic disorders, chronic gastrointestinal disease, immunodeficiency, malignancy, mental disabilities. patients currently using any lipid lowering drugs, or an alternative treatment to lower plasma cholesterol (such as probiotics) and individuals who have used antibitoics in the previous three months prior to study and pregnant or lactating women were excluded from the study. Participants were informed that they could withdraw the study at any time.

ETHICAL CONSIDERATIONS

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the ethics committee (with a document number; 2018/60-26). Written informed consent were voluntarily obtained from all participants and the study was registered on public clinical trials registry of U.S. National Library of Medicine Clinical Trial and had a registration ID number as NCT04701775.

INTERVENTION

Participants were randomly divided into three groups. Randomisation was carried out by utilising a random number table; for this, an independent coordinator, not otherwise involved in the study, created the allocation sequence assigning participants as following simple randomization procedures (computerized random numbers): 1) probiotic group who take once a day 1 x 10^6 colony forming unit (CFU) *Lactobacillus rhamnosus GG* microorganism (n = 18) capsule; 2) probiotic group II who take once a day a combination of *Lactobacillus acidophilus* 1 x 10^9 CFU and *Bifidobacterium animalis* subsp.*lactis* 1 x 10^9 CFU probiotic capsule (n = 17); and 3) placebo group: emptied capsule (n = 16) (Fig. 1). A total of 63 patients diagnosed with hypercholesterolemia, 51 were recruited into the study. The investigator and patients were blinded to the intervention. The code to the randomization sequence was only revealed to the main investigator after the final data analysis. An identification number

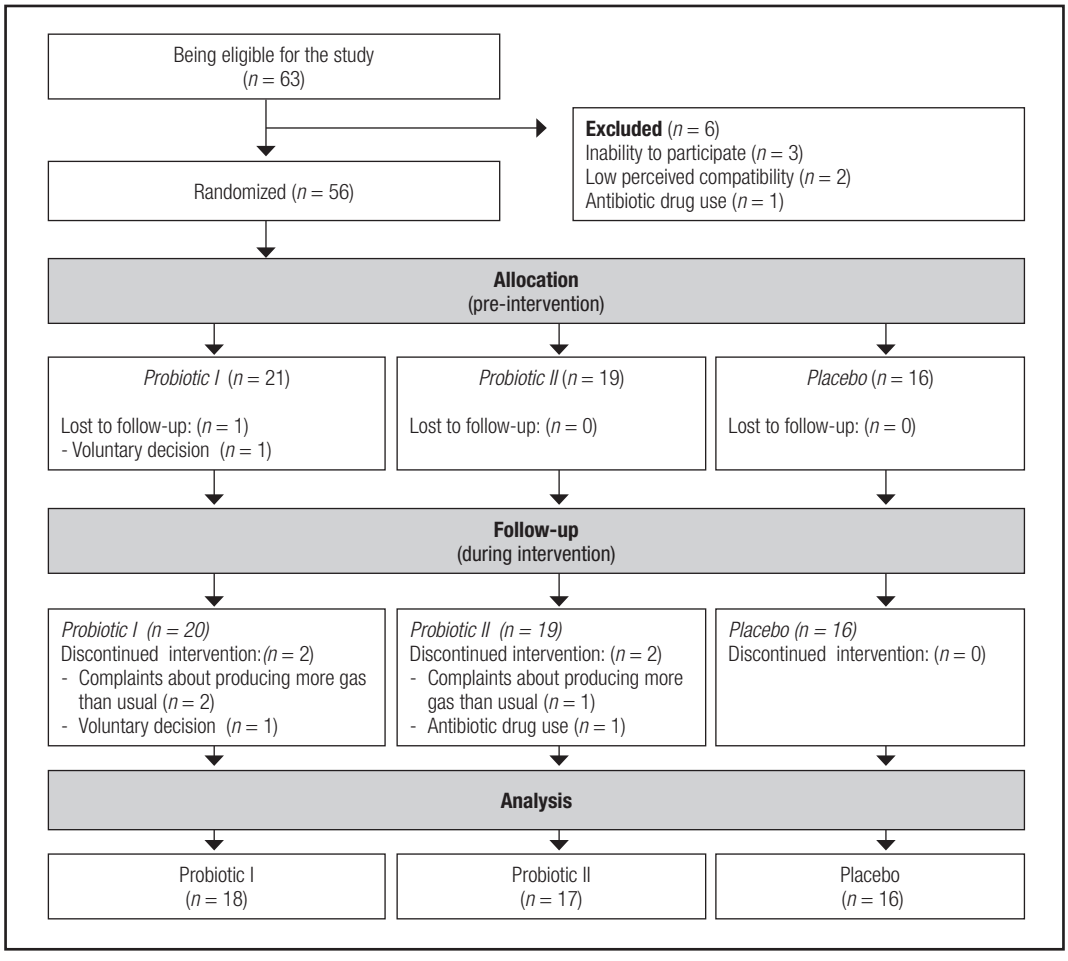

Figure 1.
Study flow chart and enrolment.

was generated for each participant and it was recorded by the pharmacist in three capsules of the product to which the person was allocated.

Pharmacist assigned participants to their group (the order of assignment was hidden by the independent coordinator until the moment of assignment), oral and written dietary instruction about their regimen was informed by independent coordinator. However, the pharmacist did not participate in data collection and/or analysis. Both participants and other research team members (with the exception of the mentioned pharmacist) were blinded to treatment allocation until the database was unlocked and data analysis was complete. Except for the interventionist (pharmacist), investigators and staff were kept blind to supplementation assignment of the participants. All investigators and participants were kept masked to outcome measurements and trial results. Study products was packed in duly labeled package and also in order to preserve the study blinding, identical placebo capsules were used to matched for size, shape, colour, texture and packaging which and consecutively numbered for each participants according to the randomisation schedule. One capsule a day were asked to be consumed by the recruited participants for the study duration and in order to ensure that all participants consume the product under the same conditions, it is requested to be consumed immediately after breakfast. Each participant was assigned an order number and received the capsules in the corresponding prepacked packages. All participants were informed of the risks and benefits of the study and were aware that they could leave the study at any time and for any reason.

ANTHROPOMETRY AND BODY COMPOSITION

All anthropometric measurements were reported by a dietitian according to the method described by Lohman et al. (17). Body weight and percentage of body fat were measured using a body composition analyser (Bioimpedance analyzer; Tanita-BC 420 s). Participants' body composition was measured while they were wearing light clothing and were barefoot with bare hands, with a precision of 0.5 kg. Height was measured using a stadiometre with 0.5 cm precision in a normal standing position without shoes. BMI was calculated as BW/height (m^2) and waist circumference (WC) were measured at the midpoint between the iliac crest and the lower rib while standing. All participants were instructed to fast for 12 h (an overnight fast). the participants were also instructed to avoid exercising, consuming alcohol for 48 h before the test. Moreover, 30 min before, the test participants were asked to fully urinate and not to consume water. A senior investigator performed these measurements before and after the intervention.

PLASMA MEASUREMENTS

At the beginning and end of the study, blood samples were drawn into vacutainer tubes containing Na2EDTA (1 g/L final concentration) from the antecubital vein after an overnight fast. The tubes were then immediately stored into ice water. Within 2 h, plasma was seperated by centrifugation at 2500 g for 20 min at 4 °C**.** All the measurments were made immediately after the plasma collection. Glucose concentrations were measured by glucose oxidase and peroxidase reactions. Total cholesterol was measured by cholesterol esterase, cholesterol oxidase and peroxidase reactions. Total TAG was measured by glycerol-phosphate-oxidase and peroxidase reactions. The method for direct determination of HDL-cholesterol uses polyethylene glycol (PEG) based system in which sulfated α-cyclodextrin, dextran sulfate, and $MgCl^2$ form water soluble complexes with the non-HDL lipoproteins present in a sample, after which pegylated cholesterol esterase and cholesterol oxidase are introduced. LDL cholesterol concentration were calculated using the Friedewald formula: (total cholesterol) - (HDL cholesterol) - (VLDL cholesterol) = LDL cholesterol. VLDL cholesterol concentrations were estimated as TAG divided by 5 when concentrations are expressed in mg/dL (18).

STATISTICAL ANALYSIS

Estimation of an appropriate sample size was conducted using the G*Power analysis method. The power value of the study was calculated as 96 % with an effect size of 0.5. Rationale for sample size was based on a previous study evaluating different probiotic capsules in hyperlipidemic patients. This study revealed an effect size for blood cholesterol of 0.5 after the probiotic capsule ingestion. A sample size of fifteen per group was determined with an effect size of 0.5, and 80 % power at the predetermined level of $\alpha = 0.05$. To account for potential subject attrition, it was planned to recruit an extra five participants per group, which increased the final sample size to twenty participants per group. Main statistical analysis were analyzed by statistical analytical systems software (package 20.0). The normality of data was confirmed using One-Sample Kolmogrow-Smirnov test. The mean ± SD were determined, and the differences among baseline, control diet, and probiotic groups were compared by analysis of paired sample t-test. Pearson correlation test was used because of the normal distribution of the data set from the relationships between the biomarkers. Nutrient intake (total fat, saturated fatty acid (SFA), mono-unsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) were also compared with the changes of plasma lipid concentrations. Also, chi square was used in order to make an assumption and compare the two qualitative (categorical) variables. The level of significance was considered $p < 0.05$.

RESULTS

As shown in figure 1, in total 51 hypercholesterolemic subjects completed the trial as detailed in the study protocol. A total 51 patients were allocated into three groups: 18 participants allocated in the probiotic group I (only *Lactobacillus*), 17 participants

allocated in the probiotic group II (combined *Lactobacillus* and *Bifidobacterium*), and 16 participants were allocated in the the placebo group. Before the randomisation, 6 subject declined to participate in the study becuase of the inability to participate, low perceived compatibility and antibiotic drug use. A total of 56 patients were allocated into 3 study groups, 56 of the 51 patients completed the assigned protocol. Table I shows the demographic characteristics of the participants in all 3 groups who provided outcome measures. Mean age was 46 years (SD = 8) for the only *Lactobacillus* group, 44 years (SD = 9) for combination of *Lactobacillus* and *Bifidobacterium* group, and 45 years (SD = 8) for the placebo group. Most participants in all three groups were female and it has also been shown that the ratio of men to women in the 3 study groups was similar. As seen in table I, all groups were matched according to their age and sex.

Body composition measurements for pre-interveniton and post-intervemtion are shown in table II. During the study period, no statistically significant difference was found between the body composition measurements of the participants in all groups ($p < 0.05$). Participants' body composition was closely monitored throughout the study, as body weight is an important factor that may influence the outcome of the study and biochemical biomarkers. Body composition measurements, especially body weight, BMI, and body fat may affect serum plasma lipids and other biochemical biomarkers. As seen in table II, there was no statistically significant difference between body composition measurements taken at the beginning and at the end of the study in all groups ($p < 0.05$).

Participants´ nutrient intake during the study is shown in table III. As shown in table III, the energy intake of the participants in the probiotic group II is statistically higher than the other 2 study groups ($p = 0.03$). Consequently, as expected, the high energy intake has led to a parallel increase in macronutrient intake. Thus, the nutrient intake of participants in this group was found to be significantly higher than the other two groups.

Mean baseline, final and change in serum lipids levels in study groups are seen in table IV. Also, figure 2 shows the change in serum lipids of participants with probiotic intervention. Baseline evaluation (pre-intervention) revealed no differences among study groups except triglyceride levels. Among the groups, the initial triglyceride levels were compared and it was determined that the mean triglyceride levels in group I was significantly higher than the other groups. There was a statistically significant difference especially between the I group and the placebo group ($p = 0.02$). As seen in table IV, after the intervention period, participants of both group who used the probiotic capsules showed a statictically signifcant reduction in total cholestrol and triglyceride ($p < 0.05$) than the group that received a placebo.

Mean baseline, final and change in fasting blood glucose values are shown in table V. There was a statistically significant decrease in fasting blood glucose level ($p = 0.000$) of both probiotic groups. Regarding the mean baseline and final changes in hs-CRP and homocysteine levels, there was a statistically significant mean difference in the probiotic group using the combined capsule, as seen in table V. However, there were no significant changes in the *Lactobacillus* or placebo group.

Table I. Demographic characteristics of the participants

Measures	Probiotic I (*n* = 18)		Probiotic II (*n* = 17)		Placebo (*n* = 16)		Total (*n* = 51)	
	n	%	n	%	n	%	n	%
Gender n (%)								
Female	11	61.1	11	64.71	11	68.75	33	64.71
Male	7	38.89	6	35.29	5	31.25	18	35.29
Age, yr	46.78 ± 8.42		44.12 ± 9.07		45.62 ± 6.84		45.53 ± 8.11	

Table II. Initial and final body composition measurements of participants

Measures	Group	Pre-intervention			Post-intervention			
		$\bar{\chi} \pm s$	p_1	Difference	$\bar{\chi} \pm s$	p_2	Difference	p_3
Body weight (kg)	Probiotic I	69.62 ± 11.74	0.153		69.93 ± 11.89	0.128		0.103
	Probiotic II	77.84 ± 12.83			78.02 ± 12.68			0.492
	Placebo	71.38 ± 6.86			71.64 ± 6.72			0.338
Height (cm)	Probiotic I	166.44 ± 9.18	0.074		166.44 ± 9.18	0.074		1.000
	Probiotic II	173 ± 9.12			173 ± 9.12			1.000
	Placebo	167.06 ± 9.42			167.06 ± 9.42			1.000

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Table II (cont.). Initial and final body composition measurements of participants

Measures	Group	Pre-intervention			Post-intervention			
		$\bar{\chi} \pm s$	p_1	Difference	$\bar{\chi} \pm s$	p_2	Difference	p_3
BMI (kg/m^2)	Probiotic I	27.51 ± 6.93	0.848		27.87 ± 6.66	0.721		0.080
	Probiotic II	26.23 ± 7.67			26.42 ± 7.28			0.297
	Placebo	27.54 ± 8.16			27.83 ± 7.99			0.178
Waist circumference (cm)	Probiotic I	82.33 ± 14.06	0.606		82.44 ± 14.03	0.638		0.157
	Probiotic II	85.29 ± 9.12			85.44 ± 9.21			0.317
	Placebo	83.06 ± 7.38			83.13 ± 7.65			0.563
Hip circumference (cm)	Probiotic I	100.17 ± 10.36	0.430		100.17 ± 10.36	0.430		1.000
	Probiotic II	101 ± 6.41			101 ± 6.41			1.000
	Placebo	101.69 ± 6.53			101.69 ± 6.53			1.000
Waist/hip ratio	Probiotic I	0.82 ± 0.07	0.731		0.82 ± 0.07	0.731		1.000
	Probiotic II	0.83 ± 0.07			0.83 ± 0.07			1.000
	Placebo	0.81 ± 0.06			0.81 ± 0.06			1.000
Body fat (kg)	Probiotic I	19.09 ± 5.22	0.691		19.26 ± 5.16	0.793		0.250
	Probiotic II	20.35 ± 6.69			20.73 ± 6.82			0.080
	Placebo	21.06 ± 5.07			21.62 ± 5.18			0.010*
FFM (fat free mass)	Probiotic I	50.57 ± 10.58	0.042*	1-2[a]	50.19 ± 10.4	0.059		0.039*
	Probiotic II	57.56 ± 10.7		2-3[c]	57.23 ± 10.7			0.256
	Placebo	49.59 ± 7.53			49.38 ± 7.6			0.364
TBW %	Probiotic I	50.4 ± 4.88	0.370		49.91 ± 4.61	0.391		0.017*
	Probiotic II	51.26 ± 4.94			50.95 ± 5.2			0.087
	Placebo	48.75 ± 4.48			48.34 ± 4.62			0.011*

BMI: body mass index; TBW: total body water. Values are means ± s.d., $n = 51$. p_1*: differences among study groups in pre-intervention period;* p_2*: differences among study groups in post intervention period;* p_3*: differences among pre and post study .* [a]*Statistical difference between group 1-2.* [b]*Statistical difference between groups 1-3.* [c]*Statistical difference between groups 2-3. For* p_1 *and* p_2*: independent t-test, and for* p_3*: paired sample t-test was used. The level of significance was* $p < 0.05$.

Table III. Nutrient composition of the study groups during the study period

Measures	Group	Pre-intervention $\bar{\chi} \pm s$	p_1		Post-intervention $\bar{\chi} \pm s$	p_2		p_3
Energy (kcal)	Probiotic I	1474.66 ± 279.87	0.003*	1-2[a]	1487.46 ± 272.37	0.007*	1-2[a]	0.500
	Probiotic II	2137.47 ± 1203.33		2-3[c]	2138.7 ± 1197.44		2-3[c]	0.619
	Placebo	1456.13 ± 289.8			1527.64 ± 281.71			0.039*
Protein (g)	Probiotic I	76.55 ± 16.25	0.801		75.41 ± 19.31	0.054		0.446
	Probiotic II	94.55 ± 55.32			105.46 ± 56.54			0.005*
	Placebo	77.17 ± 16.45			76.76 ± 17.67			0.796
Fat (g)	Probiotic I	52.52 ± 9.66	0.009*	1-2[a]	53.52 ± 10.66	0.042*	1-2[a]	0.360
	Probiotic II	75.13 ± 40.95		2-3[c]	73.41 ± 40.09		2-3[c]	0.287
	Placebo	51.84 ± 9.9			55.22 ± 10.46			0.179
Carbohydrate (g)	Probiotic I	164.94 ± 39.27	0.001*	1-2[a]	167.8 ± 35.12	0.011*	1-2[a]	0.616
	Probiotic II	254.68 ± 143.93		2-3[c]	251.31 ± 144.87		2-3[c]	0.287
	Placebo	161.2 ± 39.78			172.06 ± 39.86			0.179

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Table III (cont.). Nutrient composition of the study groups during the study period

Measures	Group	Pre-intervention $\bar{\chi} \pm s$	p_1		Post-intervention $\bar{\chi} \pm s$	p_2		p_3
Fiber (g)	Probiotic I	30.33 ± 12.97	0.158		26.07 ± 10.23	0.001*	1-2[a]	0.557
	Probiotic II	41.96 ± 31.01			37.79 ± 17.44		2-3[c]	0.723
	Placebo	29.1 ± 11.6			28.37 ± 10.56			0.756
Saturated fat (g)	Probiotic I	12.92 ± 2.93	0.378		14.40 ± 2.93	0.226		0.094
	Probiotic II	17.55 ± 9.09			20.66 ± 14.36			0.136
	Placebo	13.05 ± 2.95			14.98 ± 2.87			0.020*
Mono-unsaturated fat (g)	Probiotic I	13.36 ± 2.83	0.073		12.82 ± 3.58	0.036*	1-2[a]	0.528
	Probiotic II	18.94 ± 10.2			19.08 ± 10.9		2-3[c]	0.906
	Placebo	13.55 ± 2.92			13.66 ± 3.3			0.836
Polyunsaturated fat (g)	Probiotic I	16.03 ± 7.65	0.022*	1-2[a]	15.09 ± 8.23	0.235		0.102
	Probiotic II	24.63 ± 13.26		2-3[c]	21.46 ± 14.43			0.124
	Placebo	16.11 ± 7.67			16.28 ± 8.62			0.352
Dietary cholesterol (mg)	Probiotic I	213.82 ± 99.89	0.952		198 ± 83.42	0.586		0.446
	Probiotic II	213.59 ± 115.35			281.55 ± 222.41			0.039*
	Placebo	214.92 ± 82.42			196.65 ± 69.32			0.438
Omega 3 (g)	Probiotic I	2.35 ± 1.58	0.331		2.42 ± 1.72	0.516		0.794
	Probiotic II	3.04 ± 1.48			2.83 ± 1.34			0.962
	Placebo	2.39 ± 1.53			2.52 ± 1.71			0.856
Omega 6 (g)	Probiotic I	13.44 ± 6.36	0.019*	1-2[a]	12.53 ± 6.87	0.244		0.133
	Probiotic II	19.47 ± 6.28		2-3[c]	16.42 ± 7.17			0.068
	Placebo	13.71 ± 6.24			13.6 ± 7.32			0.255

p_1: *differences among study groups in pre-intervention period;* p_2: *differences among study groups in post intervention period;* p_3: *differences among pre and post study.* [a]*Statistical difference between group 1-2.* [b]*Statistical difference between groups 1-3;* [c]*Statistical difference between groups 2-3. For* p_1 *and* p_2*: independent t-test, and for* p_3*: paired sample t-test was used. The level of significance was* $p < 0.05$.

Table IV. Mean baseline and final change in serum lipids levels in study groups

	Group	Pre-intervention		Post-intervention		p_3	Diff.	95 % CI	
		$\bar{\chi} \pm s$	p_1	$\bar{\chi} \pm s$	p_2			Low	Up
Cholesterol	Probiotic I	241 ± 37.22		215.39 ± 42.86		0.001*	-25.61	-40.22	-11.00
	Probiotic II	226 ± 30.74	0.232	201.47 ± 31.18	0.455	0.002*	-24.53	-35.83	-13.23
	Placebo	221.63 ± 34.81		215.31 ± 33.63		0.088	-6.31	-13.07	0.45
LDL-cholesterol	Probiotic I	156.5 ± 36.08		156 ± 40.51		0.862	-0.50	-10.97	9.97
	Probiotic II	143.35 ± 29.03	0.168	137.82 ± 30.4	0.23	0.142	-5.53	-14.51	3.45
	Placebo	136.19 ± 37.1		134.75 ± 33.78		0.451	-1.44	-7.04	4.16
HDL-cholesterol	Probiotic I	57.67 ± 13.61		56.22 ± 13.33		0.2	-1.44	-4.04	1.15
	Probiotic II	63.41 ± 9.64	0.287	59 ± 10.95	0.819	0.09	-4.41	-7.29	-1.54
	Placebo	59.81 ± 12.5		59.19 ± 11.43		0.815	-0.63	-4.07	2.82

(Continues on next page)

Table IV (cont.). Mean baseline and final change in serum lipids levels in study groups

	Group	Pre-intervention		Post-intervention		p_3	Diff.	95 % CI	
		$\bar{\chi} \pm s$	p_1	$\bar{\chi} \pm s$	p_2			Low	Up
Triglyceride	Probiotic I	151 ± 72.1	0.002*	123.89 ± 69.78	0.451	0.004*	-27.11	-44.11	-10.12
	Probiotic II	121.71 ± 37.61		96.82 ± 29.9		0.006*	-24.88	-39.84	-9.92
	Placebo	83.81 ± 21.03		88.81 ± 26.84		0.776	5.00	-5.61	15.61
HDL:LDL	Probiotic I	2.9 ± 1.08	0.189	2.98 ± 1.19	0.125	0.661	0.08	-0.13	0.28
	Probiotic II	2.29 ± 0.49		2.38 ± 0.56		0.174	0.09	-0.05	0.23
	Placebo	2.36 ± 0.67		2.33 ± 0.61		0.949	-0.03	-0.22	0.16
Total:HDL	Probiotic I	4.43 ± 1.39	0.204	4.06 ± 1.39	0.456	0.014*	-0.37	-0.71	-0.03
	Probiotic II	3.61 ± 0.55		3.48 ± 0.58		0.171	-0.13	-0.30	0.04
	Placebo	3.82 ± 0.76		3.74 ± 0.69		0.801	-0.08	-0.37	0.20

LDL: low-density lipoprotein; HDL: high density lipoprotein. p_1*: differences among study groups in pre-intervention period;* p_2*: differences among study groups in post intervention period;* p_3*: differences among pre and post study. For* p_1 *and* p_2*: independent t-test, and for* p_3*: pairedsample t-test was used. The level of significance was* $p < 0.05$.

Table V. Mean baseline and final change in gylycemic and other biomarkers in study groups

	Group	Pre-intervention		Post-intervention		p_3	Diff.	%95 CI	
		$\bar{\chi} \pm s$	p_1	$\bar{\chi} \pm s$	p_2			Low	Up
FBG	Probiotic I	97.89 ± 7.42	0.956	87.61 ± 6.39	0.188	0.000*	-10.28	-13.78	-6.77
	Probiotic II	100.76 ± 18.55		90.59 ± 11.81		0.000*	-10.18	-16.23	-4.12
	Placebo	97.06 ± 8.24		92.06 ± 6.77		0.003*	-5.00	-9.09	-0.91
CRP	Probiotic I	0.24 ± 0.22	0.519	0.23 ± 0.17	0.014*	0.777	-0.01	-0.12	0.11
	Probiotic II	0.25 ± 0.22		0.28 ± 0.26		0.410	0.02	-0.03	0.07
	Placebo	0.19 ± 0.16		0.18 ± 0.13		0.280	-0.04	-0.08	0.00
Homosis-tein	Probiotic I	13.76 ± 8.6	0.089	11.77 ± 6.37	0.235	0.085	-1.98	-3.69	-0.28
	Probiotic II	9.68 ± 3.03		8.78 ± 2.78		0.044*	-0.89	-1.61	-0.18
	Placebo	8.83 ± 2.11		9.64 ± 2.13		0.006*	0.81	0.30	1.32
Insulin	Probiotic I	10.24 ± 6.05	0.583	9.52 ± 3.86	0.402	0.372	-0.72	-4.45	-1.57
	Probiotic II	9.56 ± 5.4		8.47 ± 3.54		0.277	-1.09	-3.83	-2.24
	Placebo	8.01 ± 4.64		7.91 ± 3.97		0.877	-0.1	-3.91	-3.72
HOMA-IR	Probiotic I	2.48 ± 0.55	0.756	2.05 ± 0.59	0.384	0.004*	-0.43	-0.34	-0.27
	Probiotic II	2.37 ± 0.48		1.89 ± 0.28		0.002*	-0.48	-0.26	-0.17
	Placebo	1.91 ± 0.32		1.80 ± 0.27		0.089	-0.11	-0.44	-0.15

fbg: fasting blood glucose. p_1*: differences amon gstudy groups in pre-intervention period;* p_2*: differences among study groups in post intervention period;* p_3*: differences among pre and post study. For* p_1 *and p2: independent t-test, and for* p_3*: paired sample t-test was used. The level of significance was* $p < 0.05$.

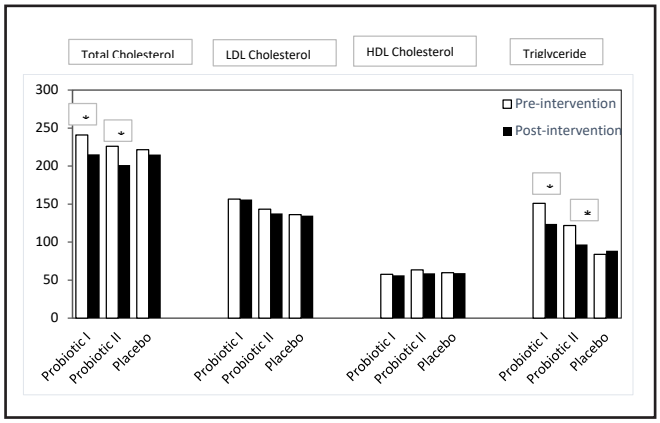

Figure 2. Effects of 8 weeks of different probiotic consumption on serum lipids compared with placebo (*$p < 0.05$ statistically significant difference).

DISCUSSION

Current double blind randomized controlled study demonstrated that 1 x 10^6 colony forming unit (CFU) *Lactobacillus rhamnosus GG* microorganism and combination of 1 x 10^9 CFU *Lactobacillus acidophilus* and 1 x 10^9 CFU *Bifidobacterium animalis* subsp. *lactis* both probiotic capsules over eight weeks had efficient role in lowering serum total cholesterol and triglyceride levels as well as showing an efficient reduction on fasting plasma glucose level, insulin and HOMA-IR levels in patients with hypercholesterolemia. Contrary to this finding, in the plasebo group there was no significant decrease was observed in plasma lipids and plasma glucose levels. Current study findings mostly indicated parallel results with the majority of previous studies evaluating the cholesterol lowering effects of probiotics (19-21). Most of the previous studies have shown that probiotic capsules have a lowering effect especially on total cholesterol and LDL cholesterol, but many of the same studies have demonstrated that probiotic use does not affect triglyceride levels (19-23). Unlike other studies, the two different types of probiotics used in the current study significantly reduced total cholesterol and especially triglyceride levels, while not having any effect on LDL cholesterol. A current new research revealed the similar findings and strenghten the current study results (24). Jaff et al. compared body composition, serum lipid and serum glucose levels between groups receiving probiotics and those not receiving probiotics. According to the Jaff et al. study results, although there was a decreasing trend in all serum lipids, no statistically significant decrease was shown, including LDL cholesterol levels. However, although a decrease in LDL cholesterol was not found to be statistically significant in this study, statistically significant decreases were detected in the average total cholesterol and triglyceride levels of participants using probiotics (probiotic I and probiotic groups II), respectively 26 mg/dL (10.7 %) and 25 mg/dL (11.1 %). While the triglyceride levels of the participants in the probiotic group I decreased by an average of 18.5 % compared to the pre-intervention, this rate was found to be 20.6 % in the probiotic group II. In the placebo group, no significant decreases in total cholesterol and triglyceride levels were observed during the study. The study of Ahn et al. (25), which supports the findings of the present study, provides parallel results; reported a decrease in triglyceride levels of 18.3 % in patients with hypertriglyceridemia who took probiotics as a combination of *Lactobacillus plantarum* and *Lactobacillus curvatus* for 12 weeks. Contrary to some previous clinical studies (19-23), an expected decrease in LDL cholesterol (LDL-C) was not detected in the current study. Although there was a tendency for a decrease in LDL-C levels of the participants using combination of *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis* (probiotic group II), no significant decrease was determined in all 3 study groups ($p > 0.05$). As mentioned in some previous studies (26,27), it has been stated that for the therapeutic effect of probiotics, the intervention should be longer than 6 weeks if possible, and thus a stronger lowering effect on LDL-C can be observed with longer interventions. As Jiang et al. (26) showed in their meta-analysis, the duration of probiotic supplementation was positively associated with the LDL-C lowering effects of probiotics. This is consistent with the results of a recent review (27), which indicated that trials lasting longer than 8 weeks had more significant lowering effects on LDL-C than those shorter than 8 weeks (27).

In addition to this, it is thought that another reason why the decrease in LDL cholesterol is not significant may be related to the probiotic dose used. In previous studies, participants taking higher doses of probiotics showed greater effectiveness on LDL-C. As Jiang et al. (2020) indicated in their metaanalyses (26), high-dose probiotics more effectively reduced LDL-C levels than low-dose probiotics. Additionally, another study by Zhang et al. (28) found that the survival of probiotic strains through the gastrointestinal passage is a key requirement for the efficacy of probiotics. Since some probiotic strains may have a low survival rate, the administration of high-dose probiotics can maximize the likelihood of gut colonization (28). Jiang et al. underlined that subgroup analyses made according to probiotic dosage showed that probiotic supplementation was only effective in reducing LDL-C levels when the dosage exceeded 10^9 CFU/day. In contrast, no changes in LDL-C levels were observed in patients given dosages below 10^9 CFU/day (26). In this study, while no decrease was observed in LDL-C levels in probiotic group I, a decrease was detected in probiotic group II, probably because the dosage used by participants was 10^9 CFU/day. However, it is thought that the reason why the decrease is not statiscally significant is likely related to the study duration.

Based on *in vitro* studies, the effect of probiotics in lowering plasma cholesterol is associated with different mechanisms; such as assimilation of cholesterol during growth by *L. Acidophilus,* binding of cholesterol to the cellular surface (29,30,) disruption of cholesterol micelles (29), and deconjugation of bile salt and bile salt hydrolase activity (30,31). A recent study has found that *L. acidophilus* reduces cholesterol absorption through the down-regulation of Niemann-Pick C1-like 1 in Caco-2 cells (32). While decreases in blood lipids were expected in people using probiotics through all the mentioned mechanisms, more significant decreases were expected especially in individuals in group II using combined strains. Since, studies indicated that combined strains are more effective in lowering plasma lipids than using a single strain (33,34).

Once the effect of probiotic intervention on glycemic biomarkers was evaluated, this study showed that the fasting insulin levels of group II participants who took capsules containing *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp.*lactis* decreased by 11.4 %, insulin levels of participants who took capsules containing only *Lactobacillus rhamnosus GG* (group I) decreased by 7.3 %, while this decrease was only 1.1 % in the placebo group. Similar results were observed in fasting serum glucose levels; after the intervention, there was a 12.5 % reduction in fasting serum glucose levels in probiotic group I, while a 9.5 % decrease was observed in probiotic group II. A decrease was also found in the placebo group (5.2 %), but not as significant as in probiotic intervention groups (Table IV). The present study, which has parallel findings with the meta-analysis previously compiled by

Ruan et al. (35) showed significant decreases in HOMA-IR values of probiotic supplemented participants. HOMA-IR value, which is an indicator of insulin resistance, decreased by 0.43 in probiotic group I, while a decrease of 0.48 was found in probiotic group II ($p < 0.05$) (Table IV). In this regard, the results of the present study show that both single and combined strains of probiotics, which are used regularly for 8 weeks, positively affect fasting blood glucose, fasting insulin level and therefore HOMA-IR. Similar results were obtained in a recent clinical study conducted with probiotic supplementation, and a decrease in blood glucose, insulin and HOMA-IR levels was detected in the group using probiotics compared to the placebo group (24).

It is known that high serum homocysteine levels are an independent risk factor for coronary heart disease (36). However, it is not possible to reach a definitive conclusion due to the small number of clinical studies examining the effect of probiotics on cardiovascular diseases through the homocysteine mechanism. In the present study, the effect of probiotic use on homocysteine level was evaluated and a significant decrease was associated only in the probiotic group II who were receiving the combined strain (Table IV). Parallel to this study, Valentini et al. (37) carried out a study in order to determine the effect of probiotic use on oxidative stress and inflammation biomarkers. Valentini et al., allocated participants into two groups and one group had only dietary intervention and the other group had dietary intervention plus VSL #3 probiotic strains (*Lactobacillus acidophilus, delbrueckii* subsp. *Bulgaricus, casei, plantarum, Bifidobacteria breve, B. Longum, infantis, Streptococcus salivarius* subsp. *thermophilus*). According to the results of this study, dietary intervention alone reduced fasting total cholesterol and glucose levels, while diet intervention plus probiotic supplementation has been shown to statistically improve folate, vitamin B12 and homocysteine levels. Barreto et al. (38) conducted an intervention study to examine the effect of probiotic use on homocysteine levels. During the study, participants in the intervention group ($n = 12$) were given fermented milk (80 mL/day) containing *Lactobacillus plantarum* for 90 days, while participants in the control group ($n = 12$) were given unfermented milk (80 mL/day) for 90 days and evaluation has been made. The findings of the study are consistent with those of Valentini et al. (37) and showed parallel results to the findings of the current study, showing that participants who consumed fermented milk for 90 days had a decrease in glucose and homocysteine levels compared to the control group.

The role of inflammation in the propagation of atherosclerosis and susceptibility to cardiovascular (CV) events is well established. Of the wide array of inflammatory biomarkers that have been studied, high-sensitivity C-reactive protein (hsCRP) has received the most attention for its use for the prediction of cardiovascular disease (39). Although there are studies showing that the use of probiotics reduces hs-CRP levels, some studies show that probiotic use does not affect hs-CRP levels (40-42-40). Ryan et al. (40) showed in their study in 12 individuals with hyperlipidemia that the use of 5.6 x 10^{10} *S. boulardii* for 8 weeks did not reduce hsCRP levels. This finding is in line with studies showing that probiotics have no effect on hs-CRP levels (41,42). Similar to studies showing that probiotic use has no effect on hs-CRP, this study also showed that probiotic use did not make a significant difference on hs-CRP levels compared to the baseline ($p > 0.05$) (Table IV).

There are strengths and limitations of the current study. It was a double-blind randomized controlled study conducted with good compliance to daily probiotic consumption during the study period (8 weeks). It is one of the scarce studies in which combined strain and single strain are compared at the same time. However, current study intervetion period was based on recommendations of similar studies that have been carried out in this area yet it would be significant for further studies to extend the duration of the intervention for a better understanding of the probiotic effect over time. The probiotic dose used in the current study was similar to others studies (30-34) but for therapeutic effect it would be better to use higher doses. Based on current study, it would be better for further studies to conduct the studies with a longer follow-up periods and different daily doses of probiotics.

CONCLUSION

As a conclusion, the results of this study showed that strains containing *Lactobacillus or Bifidobacterium* could be effective in hypercholesterolemic patients not treated with conventional lipid-lowering drugs and could reduce serum lipids as well as homocysteine and glycemic biomarkers. In order to determine the effectiveness of probiotics on cardiovascular disease, further studies are needed using different bacterial strains and different dosages to determine the potential role for probiotic bacteria in the management of hyperlipidaemia.

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