ORIGINAL ARTICLE

Do symbiotic and Vitamin E supplementation have favorite effects in nonalcoholic fatty liver disease? A randomized, double-blind, placebo-controlled trial

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Background: Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world. Oral administration of symbiotic and Vitamin E has been proposed as an effective treatment in NAFLD patients. This study was carried out to assess the effects of symbiotic and/or Vitamin E supplementation on liver enzymes, leptin, lipid profile, and some parameters of insulin resistance (IR) in NAFLD patients. **Materials and Methods:** We randomly assigned sixty NAFLD adult patients to receive (1) symbiotic twice daily + Vitamin E-like placebo capsule; (2) 400 IU/d Vitamin E + symbiotic-like placebo; (3) symbiotic twice daily + 400 IU/d Vitamin E; and (4) symbiotic-like placebo + Vitamin E-like placebo for 8 weeks. **Results:** Symbiotic plus Vitamin E supplementation led to a significant decrease in concentrations of liver transaminase ($P \leq 0.05$). Mean difference of apolipoprotein A-1 was more significant in symbiotic group compared to control. However, mean difference of apolipoprotein B100/A-1 was only significant in symbiotic group compared to control. At the end of the study, significant differences in total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) were seen between the symbiotic plus Vitamin E and control groups ($P < 0.001$). Furthermore, intake of symbiotic plus Vitamin E supplements led to a significant decrease in concentrations of triglycerides (TG) after the intervention. Significant differences in leptin, fasting blood sugar (FBS), and insulin levels were seen between the symbiotic plus Vitamin E and control groups at the end of the study ($P < 0.001$). In contrast, symbiotic and/or Vitamin E supplementation did not affect high-density lipoprotein cholesterol and homeostasis model assessment for IR levels. **Conclusion:** In our study, symbiotic plus Vitamin E supplementation was the most effective treatment in lowering liver enzymes, leptin, FBS, insulin, TG, TC, and LDL-C among NAFLD patients.

Key words: Leptin, lipid profile, nonalcoholic fatty liver disease, symbiotic, Vitamin E

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) comprises a spectrum of liver disorders, ranging from fatty liver alone to steatohepatitis, steatonecrosis, and nonalcoholic steatohepatitis (NASH).[1] NAFLD patients with simple steatosis are unlikely to progress to cirrhosis whereas 20% of patients with NASH are at the greatest risk of progressing to cirrhosis over 15 years as a result of inflammation and hepatocellular injury.[2] NAFLD is increasingly recognized as a serious, worldwide public health concern and considered the hepatic manifestation of metabolic syndrome or insulin resistance (IR) and expanding in line with outbreak of obesity and type 2 diabetes.[3,4]

In spite of tremendous research, the mechanisms underlying NAFLD development remain to be clarified.[5] However, IR, visceral adiposity, adipokines, oxidative stress, and increased free fatty acid (FFA) release contribute to the pathogenesis of liver steatosis.[6] Recently, increased intestinal permeability and alteration in the ratio of gut microbiota have been seen in patients with obesity and NAFLD, attributing a potential role in the pathogenesis of NAFLD.[7]

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Intestinal microflora has been first claimed to have a positive influence on human health, and the ensuing research has by now soundly confirmed this concept. Probiotics are live microorganisms that, when consumed in adequate quantities, confer a health benefit to the host.[8] In the past two decades, a number of investigators have sought to determine that probiotics along with prebiotics have anti-inflammatory, immunomodulatory, antifibrotic, and lipid-lowering properties. Besides, regarding fibrotic and steatotic characteristics of NAFLD,[9,10] it was assumed that symbiotic supplementation might act as a novel therapeutic strategy in patients with this disease.

Cytotoxic FFA oxidation in liver can upregulate cytokines production, which, in turn, induces cytochrome P450 enzyme expression and reduces liver concentration of antioxidants.[11] Furthermore, increased production of cytokines in hepatic stellate cells (HSCs) leads to fibrogenesis and extracellular matrix protein deposition.[12] Vitamin E as a fat-soluble vitamin with antioxidant properties can inhibit proinflammatory cytokine production and attenuate hepatic fibrosis production.[13]

Research on the NAFLD and probiotic supplementation has been mostly restricted to a limited number of some species of probiotics. In addition, far too little attention has been paid to synergistic effect of multiple strains of probiotic and Vitamin E supplements in NAFLD patients. The present study was, therefore, performed to investigate the synergistic effect of symbiotic and Vitamin E supplementation on some indicators such as liver enzymes, leptin, lipid profile, and glycemic status in NAFLD patients.

MATERIALS AND METHODS

Recruitment and eligibility screening

The present randomized, double-blind controlled clinical trial was conducted in Tehran, Iran, during 2012–2013 among sixty NAFLD patients (48 men and 12 women) aged 25–64 years, who were recruited from Hazrat-e-Rasoul Medical Complex (Colorectal Research Center) in Tehran, Iran, which is only devoted to gastrointestinal (GI) and liver disorders. In this double-blind, placebo-controlled trial, eligible patients signed a written consent form after a full review of the risks and benefits of the study, which was approved by the Ethical Committee of Iran University of Medical Sciences (grant number: 90005246) in 2011 and registered at the Iranian Registry of Clinical Trials (IRCT201111082709N22) and was funded by the Iran National Science Foundation. All procedures were in accordance with the guidelines in the Helsinki declaration (2013).

The diagnosis of NAFLD was based on hepatic ultrasonography (Grade 1–3), associated with persistently elevated levels of alanine aminotransferase (ALT) concentration (30 mg/dL) for 6 months before the study and at the time of randomization. The eligibility criteria included females and males between the ages of 25 and 64 years with body mass index (BMI) ranging from 25 to 35 kg/m^2.

Exclusion criteria comprised any of the pathologic conditions affecting liver such as viral hepatitis, alcohol consumption, hypothyroidism, Wilson disease, acute systemic disease, cystic fibrosis, coeliac disease, and alpha-1-antitrypsin deficiency. Patients were also excluded if they had history of cancer, metabolic disorders, cardiovascular (CVD), and autoimmune diseases as well as drug or alcohol abuse. Diabetes mellitus, pregnancy, lactation, menstruation at the time of blood sampling, infectious diseases during the study, use of nonsteroidal anti-inflammatory drugs, antibiotics, and probiotics and food supplements preceding enrolment were also considered exclusion criteria.

Study design and randomization

All eligible patients with NAFLD were recruited during 2012–2013 [Figure 1]. Each symbiotic capsule (Protexin; Probiotics International Ltd., Lopen Head, Somerset, United Kingdom) contained *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Streptococcus thermophilus*, *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Lactobacillus bulgaricus*, and prebiotic (fructooligosaccharide) and probiotic cultures (magnesium stearate [source: Mineral and vegetable] and a vegetable capsule (hydroxypropyl methylcellulose)). The concentration of each probiotic strain was 2 × 10^8 CFU/g per capsule. Symbiotic supplements were administered as two capsules per day orally after the main meal. Two identical-appearing placebo capsules (corn starch, Zahravi Pharmaceutical Co.,) were taken daily by participants assigned to either placebo or Vitamin E group. The justification for choosing this dosage was based on the earlier study.[14]

Vitamin E (RRR-α-tocopherol, Zahravi Pharmaceutical Co.,) at a daily dosage of 400 IU and similar-appearing placebo was administered orally. This chosen Vitamin E dose was similar to previous study in NAFLD patients.[15]

Randomization was carried out according to balanced block randomization procedure; participants, nutrition specialists, and outcome assessors were all blinded to the interventions into which the individuals were allocated.

Our patients were randomly assigned in the symbiotic and Vitamin E trial: 15 were assigned to receive symbiotic and Vitamin E-like placebo capsule (group Sym), 15 were assigned to receive Vitamin E and symbiotic-like placebo (group Vitamin E), 15 were assigned to symbiotic and Vitamin E supplementation (group Sym + Vitamin E), and

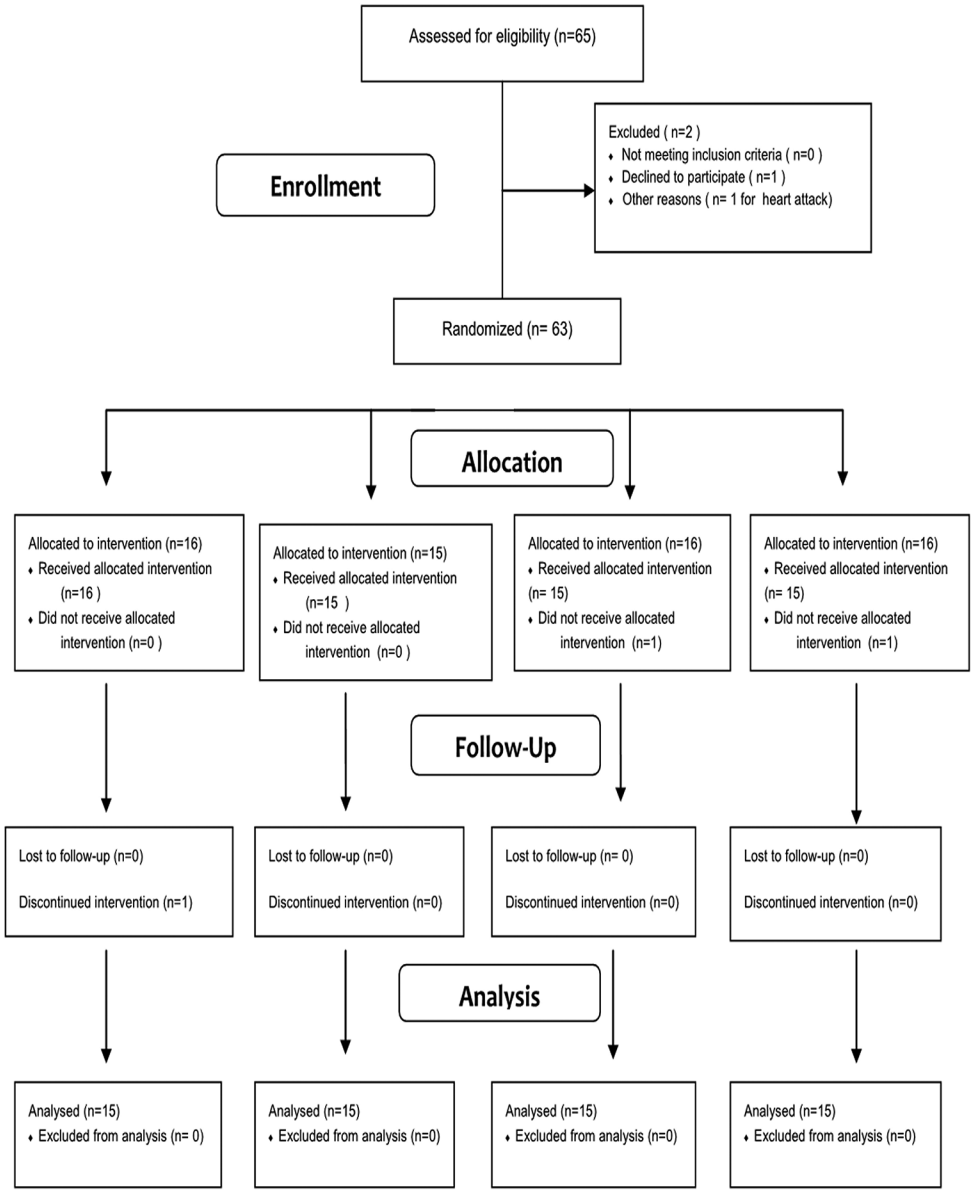

Figure 1: Flow diagram of patient recruitment and randomization process

15 were given symbiotic-like placebo and Vitamin E-like placebo supplementation (control).

At the beginning of the study, physical examinations, medical history, diet, and physical activity level of each patient were assessed. The patients were asked not to consume any probiotic containing food, yogurt, or its products during an initial 2 weeks run-in period before the dietary intervention. At the end of the run-in period, eligible patients were randomly assigned to one of the named groups. They were also asked not to consume other probiotic products during the intervention.

Compliance was monitored by phone calls weekly and verified using capsule counts (number of capsules left in the capsule bottle at the end of the study).

Clinical, paraclinical, and dietary intake assessments

The patients underwent anthropometric and laboratory assessments at the baseline and at the end of the study. Weight, height, and waist circumferences of each patient were measured according to the standard protocols. Each individual's BMI was calculated as body weight (kg)/height^2 (m).

Blood samples were obtained and analyzed after 10–12 h of overnight fasting. All blood analyses were done at the same laboratory using standard laboratory methods. ALT, aspartate aminotransferase (AST), and alkaline phosphatase (ALP) concentrations were measured by enzymatic methods (Pars Azmoon Inc, Tehran, Iran). Leptin serum concentrations were measured using a commercial ELISA kit (LDN, Nordhorn, Germany).

Total cholesterol (TC) and serum triglycerides (TG) were tested using the enzymatic colorimetric method. High-density lipoprotein cholesterol (HDL-C) measurement was done after precipitation of the apolipoprotein B-100 (apo B-100) containing lipoproteins with phosphotungstic acid. All of these analyses were performed using commercial kits (Pars Azmoon Co., Iran). Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated using the Friedewald equation: LDL-C = TC-HDL-C-TG/5.[16] Serum apolipoprotein A-1 (apo A-1) and apo B-100 concentrations were determined by immunoturbidimetric methods using commercial kits (Roche Diagnostics, Mannheim, Germany).

Serum glucose concentrations were measured using the enzymatic colorimetric method with glucose oxidase (Pars Azmoon Co., Iran). Fasting insulin concentrations were analyzed by ELISA kit (DiaMetra Co., Italy). Homeostasis model assessment for IR (HOMA-IR) as a marker of IR was calculated by the following formula: [17] HOMA-IR = (fasting insulin [mU/L] × fasting blood glucose (mg/dL))/405.

For the assessment of nutrient intakes, patients were asked to complete the 24 h food recall questionnaire, including 2 week days and 1 weekend at the baseline and at the end of the 1st and 2nd month after the intervention. Dietary intakes were then analyzed using Nutritionist IV software (First Databank Inc., Hearst Corp., San Bruno, CA, USA). The nutrient database of Nutritionist IV software was based on the United States Department of Agriculture and data related to this software were modified for Iranian foods. Physical activity was determined with the short form of the International Physical Activity Questionnaire.[18]

Statistical analysis

Data analysis was performed using SPSS software (SPSS Inc., Version 20, Chicago, IL, USA) and presented as mean ± standard deviation. The data were analyzed according to the intention-to-treat principle.

Normal distribution of data was assured using Kolmogorov–Smirnov test. Differences between groups were determined by one-way analysis of variance or Kruskal–Wallis for continuous data, and the Chi-square test for categorical data. *Post hoc* comparisons were carried out with Tukey's test. The within-group comparisons of data were performed using paired *t*-test and Wilcoxon test when appropriate. The results were considered statistically significant if $P < 0.05$.

RESULTS

Characteristics of the patients

Among 65 patients enrolled in the study, sixty of the patients completed 8 weeks of treatment and had end-of-study clinical and laboratory data. In other words, five of the participants were excluded from the statistical analysis because of pregnancy, traveling, and heart attack.

In this study, 48 men and 12 women, aged 25–64 years, completed the study and there were no significant differences among intervention groups in terms of weight, BMI, and waist circumference at study baseline. Furthermore, we found no significant differences in the weight, BMI, and waist circumference within and between all treatment groups after the intervention [Table 1].

Serum liver enzymes and leptin

A significant improvement in several measured variables in this study was seen within and between all study groups. There was a significant decrease in mean serum ALT levels in patients of group Sym and Sym + Vitamin E [Table 2]. However, the mean reduction in the Sym + Vitamin E group was greater than the control group ($P < 0.001$). No significant reduction in serum ALT levels was found in Vitamin E group compared to the control.

The differences in serum AST levels are shown in Table 2. The changes for serum AST were significant in all three groups versus control while they were more evident for Sym + Vitamin E group [Table 2]. Serum ALP concentrations significantly decreased in all groups except the control although these reductions in the Sym + Vitamin E group were greater in comparison to the control group [Table 2].

A significant reduction in the concentrations of serum leptin was observed in Sym and Sym + Vitamin E groups after the 8-week of intervention. The reduction in serum leptin levels of the Sym + Vitamin E group compared with the control was − 11.4 ± 2.76 compared with 2.54 ± 3.02 ng/mL ($P < 0.001$) [Table 2].

Lipid profile

The range of measured lipid profiles [Table 3] was not statistically different among all of the four groups at the beginning of the intervention though the amounts of several of them were significantly affected after the 8-week dietary intervention. As shown in Table 3, a significant reduction in TG and TC was observed only in Sym and Sym + Vitamin E groups and the $P < 0.001$ for TG and TC in both groups. LDL-C levels decreased significantly in all three groups except the control after the intervention. Moreover, the intake of symbiotic plus Vitamin E supplements led to a more significant decrease in LDL-C compared with control (−22 ± 10.63 vs. 1.43 ± 2.67 mg/dL, $P < 0.001$).

Just for TG and TC serum concentrations, we observed statistically significant differences between the groups after the 8-week dietary intervention ($P = 0.004$ and 0.001, respectively).

Table 1: Anthropometric indicators of nonalcoholic fatty liver disease patients in intervention groups

Characteristic	Symbiotic (*n*=15)	Vitamin E (*n*=15)	Symbiotic + Vitamin E (*n*=15)	Control (*n*=15)	*P*
Height (cm)					
Week 0	172.66±10.97*	171.73±6.48	168.6±9.33	172.93±8.27	0.529†
Week 8	-	-	-	-	-
P	-	-	-	-	
Weight (kg)					
Week 0	80.73±9.12	84.86±12.71	79.8±10.05	83.46±10.25	0.535†
Week 8	80.33±9.17	84.66±12.71	79.6±10.15	96.46±7.33	0.519
P§	0.111	0.384	0.424	0.610	
Waist circumference (cm)					
Week 0	93.6±6.52	91.73±9.91	94.93±8.33	96.46±7.33	0.438†
Week 8	93.86±6.65	91.63±10.02	94.86±8.15	96.53±7.5	0.427
P value§	0.342	0.458	0.433	0.610	
BMI (kg/m^2)					
Week 0	27.28±2.21	28.77±4.08	28.05±2.52	27.84±1.96	0.724‡
Week 8	27.14±2.26	28.7±4.06	27.98±2.65	27.8±1.98	0.684
P‖	0.213	0.575	0.959	0.563	

*All values are means±SDs; †Obtained from ANOVA; ‡Obtained from Kruskal-Wallis test; §Obtained from paired *t*-test; ‖Obtained from Wilcoxon signed-rank test. BMI = Body mass index; SDs = Standard deviations

Table 2: Serum liver enzymes, glycemic profile, and leptin in nonalcoholic fatty liver disease patients before and after intervention

Characteristic	Symbiotic (*n*=15)	Vitamin E (*n*=15)	Symbiotic + Vitamin E (*n*=15)	Control (*n*=15)	*P*
ALT (IU/L)					
Week 0	38.14±8.72*	35.73±5.83	38.14±7.77	33.88±4.49	0.27†
Week 8	31.59±9.42	32.06±7.39	25.35±7.98	38.05±6.54	0.001†
Mean difference	−6.54±7.66‖	−3.66±6.81	−12.79±3.65**	4.16±3.43	<0.001†
AST (IU/L)					
Week 0	37.95±15.34	30.53±8.01	36.84±7.3	32.04±7.06	0.048‡
Week 8	30.52±13.4	24.6±6.64	25.47±5.99	34.54±6.8	0.004‡
Mean difference	−7.43±8.58	−5.93±6.61	−11.36±4.52**	2.5±5.75	<0.001‡
ALP (IU/L)					
Week 0	148.63±26.92	158.1±36.23	147.6±14.95	150.85±21.97	0.813‡
Week 8	133.79±20.88	153.53±38.16	120.79±15.35	156.04±22.52	0.003‡
Mean difference	−14.84±12.22	−4.56±9.22	−26.8±11.1**	5.19±2.64	<0.001‡
FBS (mg/dL)					
Week 0	115.74±9.9	115.66±12.45	114.13±12.33	108±14.53	0.229‡
Week 8	104.76±7.66	113.6±12.09	98.63±7.14	114.9±13.8	<0.001‡
Mean difference	−10.97±6.54	−2.06±9.21	−15.5±8.23**	6.9±7.92	<0.001‡
Insulin (μIU/L)					
Week 0	2.24±0.88	2.09±0.41	2.5±0.7	2.13±0.46	0.306‡
Week 8	1.8±0.61	2.05±0.4	1.77±0.53	2.56±0.59	0.001‡
Mean difference	−0.44±0.34	−0.04±0.12	−0.72±0.26**	0.42±0.4	<0.001‡
HOMA-IR					
Week 0	0.63±0.20	0.58±0.15	0.56±0.16	0.57±0.16	0.843‡
Week 8	0.75±0.46	0.68±0.26	0.63±0.2	0.67±0.28	0.95‡
Mean difference	0.12±0.37	0.09±0.19	0.07±0.16	0.10±0.21	0.857‡
Leptin (ng/mL)					
Week 0	31.18±6.07	29.22±5.3	37.34±3.88	35.75±6.99	0.002‡
Week 8	29.03±4.83	28.74±4.98	25.93±4.56	38.29±6.26	<0.001‡
Mean difference	−2.15±3.98	−0.48±2.76	−11.4±2.76**	2.54±3.02	<0.001‡

*All values are means±SDs, †Obtained from ANOVA, ‡Obtained from Kruskal-Wallis test; ‖Mean difference reflects week 8 minus week 0 values, **More significant reduction in symbiotic + Vitamin E group versus control (*P*<0.05). ANOVA = Analysis of variance; ALT = Alanine aminotransferase; AST = Aspartate aminotransferase; ALP = Alkaline phosphatase; FBS = Fasting blood sugar; HOMA-IR = Homeostasis model assessment for insulin resistance; SDs = Standard deviations

In other words, compared with the control group, patients taking symbiotic plus Vitamin E had a significant decrease in TC ($P = 0.002$) and TG serum levels ($P = 0.047$). In contrast, serum concentrations of HDL-C had no significant changes

Table 3: Serum lipid profiles in nonalcoholic fatty liver disease patients before and after intervention

Characteristic	Symbiotic (*n*=15)	Vitamin E (*n*=15)	Symbiotic + Vitamin E (*n*=15)	Control (*n*=15)	*P*
Triglycerides (mg/dL)					
Week 0	187±46±20.55*	190.6±26.47	186±19.09	182.8±30.2	0.852†
Week 8	167.97±20.24	188.34±24.3	162.56±18.83	186.81±26.94	0.004†
Mean difference	−19.49±10.44‖	−2.26±6.74	−23.43±5.95**	4.01±36.97	0.001†
Total cholesterol (mg/dL)					
Week 0	194.33±24.23	193.2±23.28	185.93±21.44	194.43±15.88	0.658†
Week 8	176.8±18.89	190.13±26.16	167.3±18.79	197.26±15.32	0.001†
Mean difference	−17.53±8.91	−3.06±11.15	−18.63±10.64**	2.83±4.19	<0.001†
HDL-C (mg/dL)					
Week 0	46.13±30.27	43.43±10.48	41.32±13.67	38.21±8.31	0.512‡
Week 8	48.33±28.66	45.6±7.78	43.95±9.42	38.52±8.17	0.17‡
Mean difference	2.2±7.05	2.16±9.85	2.63±10.44	0.3±7.14	0.461‡
LDL-C (mg/dL)					
Week 0	139.99±40.5	134.96±10.66	140.86±11.63	138.33±15.06	0.901†
Week 8	124.11±37.32	131.5±10.22	118.86±15.54	139.76±14.78	0.06†
Mean difference	−15.88±8.71	−3.46±3.5	−22±10.63**	1.43±2.67	<0.001†
Apo A-1 (mg/dL)					
Week 0	123.93±20.72	128.4±21.53	121.06±17.69	125.53±18.53	0.78†
Week 8	130.4±19.37	133.13±16.2	127.33±16.08	120.06±17.93	0.209†
Mean difference	6.46±7.48††	4.73±9.6	6.26±8.37	−5.46±5.16	<0.001†
Apo B-100 (mg/dL)					
Week 0	91.86±9.85	87.6±11.03	87.86±18.21	89.53±9.61	0.78†
Week 8	81.46±12.92	81.6±10.06	85.73±18.2	84.26±7.23	0.75†
Mean difference	−10.4±7.56	−6±3.76	−2.13±19.73	−5.26±7.17	0.264†
Apo B-100/A-1					
Week 0	0.75±0.12	0.69±0.11	0.73±0.16	0.72±0.09	0.655†
Week 8	0.63±0.13	0.61±0.09	0.67±0.14	0.71±0.08	0.123†
Mean difference	−0.12±0.07††	−0.07±0.05	−0.05±0.16	−0.01±0.07	0.045†

*All values are means±SDs; †Obtained from ANOVA, ‡Obtained from Kruskal-Wallis test; ‖Mean difference reflects week 8 minus week 0 values, **More significant reduction in symbiotic + Vitamin E group versus control (*P*<0.05), ††More significant reduction in symbiotic group versus control (*P*<0.05). ANOVA = Analysis of variance; HDL-C = High-density lipoprotein cholesterol; LDL-C = Low-density lipoprotein cholesterol; Apo A-1 = Apolipoprotein A-1; Apo B-100 = Apolipoprotein B-100; Apo B-100/A-1 = Apolipoprotein B-100/A-1; SDs = Standard deviations

between and within of each group after the intervention [Table 3].

At the end of the 8 weeks treatment period, apo A-1 significantly increased in group Sym (P = 0.005) and Sym + Vitamin E (P = 0.012). Mean changes of apo A-1 from baseline were more significant for Sym group. On the other hand, significant reduction in apo B-100 and apo B-100 to apo A-1 ratio (apo B100/A-1 ratio) was observed in Sym and Vitamin E group ($P \leq 0.001$) after the intervention compared to baseline. The reduction in apo B100/A-1 was only significant in Sym group compared with control (−0.12 ± 0.07 vs. −0.01 ± 0.07 mg/dL, P = 0.003).

Fasting blood sugar, insulin, and homeostasis model assessment for insulin resistance

As shown in Table 2, fasting blood sugar (FBS) and serum insulin concentrations decreased significantly in Sym and Sym + Vitamin E groups after intervention (P = 0.001). The reductions in FBS and insulin levels of the Sym + Vitamin E group compared with the control were as follows: FBS decreased as much as − 15.5 ± 8.23 compared with 6.9 ± 7.92 mg/dL ($P < 0.001$) and insulin − 0.72 ± 0.26 compared with 0.42 ± 0.4 μIU/mL ($P < 0.001$). These reductions were significant between four groups after 8-week of intervention ($P < 0.001$) [Table 2].

In contrast, when comparing all treatment groups, there was no significant difference between four groups on HOMA-IR in our study [Table 2].

Assessment of energy intake and physical activity

No significant differences were observed in reported dietary energy intake measured in calories, nutrients, and vitamins intake between and/or within the four groups at baseline and at the end of the study. In addition, physical activity was unchanged throughout the study.

No adverse effects were reported with our symbiotic mixture and/or Vitamin E supplement consumption versus placebo treatment.

DISCUSSION

In the present randomized controlled clinical trial, it was shown for the first time that a daily supplementation with symbiotic plus Vitamin E for 8 weeks was the most effective treatment in lowering levels of serum liver enzymes, some markers of lipid profile, serum leptin, FBS concentrations, and insulin in NAFLD patients. The findings in this survey are the confirmation of the recent studies.

The intestinal mucosa as a defense barrier prevents the penetration and the systemic spread of bacteria and endotoxins, most of which are lipopolysaccharides (LPS). However, failing in this barrier under certain conditions leads to bacterial and endotoxin invasion into the GI tract, systemic organs, and tissues. These microbial products exert proinflammatory actions.[19] Baldwin showed that activation of TLR4 by LPS has a distinct effect in promoting inflammation and injury in conditions such as alcoholic liver disease and NASH.[20] On the other hand, small bacterial overgrowth (SIBO) which is present in 50% of patients with nonalcoholic steatosis results in tight junction disruption and increased gut permeability, which may impact on NAFLD progression.[21]

The first question in this study was meant to determine the synergistic effect of symbiotic and Vitamin E supplementation on changes of liver enzyme levels. NAFLD as the most common cause of chronic liver disease is often identified by asymptomatic elevation of liver enzymes, especially serum ALT levels.[22] In the present study, joint symbiotic-Vitamin E supplementation for 8 weeks led to significant reduction in serum ALT, AST, and ALP levels. In accordance with our result, Eslamparast *et al*. in a randomized, double-blind study in NAFLD patients found that supplementation with symbiotic, in addition to lifestyle modification for 28 weeks, can decrease serum ALT and AST levels.[23] In a study by Aller *et al*., significant reduction in serum ALT and AST levels was observed following *L. bulgaricus* and *S. thermophilus* supplementation in NAFLD patients.[14] Similarly, VSL#3 supplementation in NAFLD patients significantly decreased these variables.[9] In line with these findings, symbiotic plus Vitamin E supplementation had more significant reduction in ALT, AST, and ALP levels in our study. Collectively, symbiotics exert their beneficial effects on liver functions and enzymes through manipulation of enteric flora, enhancing the barrier function of epithelial cells, decreasing intestinal permeability, and endotoxemia in patients with liver diseases.[24]

To the best of our knowledge, only a few studies assessed the efficacy of Vitamin E in NAFLD adult patients. In the PIVENS trial, 247 nondiabetic and noncirrhotic adults with NASH received Vitamin E (800 IU/day), pioglitazone (30 mg/day), or placebo for 96 weeks. Compared with the placebo, Vitamin E led to a robust improvement in NASH.[25] Furthermore, in one meta-analysis, Sato *et al*. showed beneficial effects of Vitamin E supplementation on liver function and histologic changes in patients with NAFLD/NASH.[26]

Increased hepatic uptake and synthesis of FFAs led to increasing generation of reactive oxygen species (ROS).[27] ROS overproduction enhances lipid peroxidation and some cytokines production, such as tumor necrosis factor-alpha (TNF-α) and transforming growth factor-β (TGF-β).[28] Lipid peroxidation and cytokine production generate more ROS production which have deleterious effects on HSCs. On the other hand, animal studies have shown that Vitamin E as a fat-soluble vitamin can decrease liver enzymes, diminish histological steatosis, and necroinflammation. Interestingly, Vitamin E downregulates the expression of TGF-β1, a cytokine implicated in the development of liver fibrosis.[29]

We also studied the effects of Vitamin E supplementation in combination with symbiotic on serum leptin levels. Leptin levels increase along with increasing fat mass as a compensatory mechanism, that limits the expansion of fat mass, and preserve insulin sensitivity. Expansion of adipose tissue as a result of obesity leads to detrimental effects of leptin, by acting as proinflammatory and profibrogenic adipokine.[30] Under some conditions, activated HSCs may produce leptin *in vivo* and express LepRb, which activation leads to increased expression of proinflammatory and proangiogenic cytokines and growth factors so triggering or augmenting fibrogenesis.[31] Besides, fatty acids and high-fat feeding can, even in the absence of obesity or systemic IR, induce TLRs that in turn trigger inflammation.[32] Some studies reported higher leptin levels in NAFLD patients[33] while others reported similar levels in NAFLD and controls.[34]

To the best of our knowledge, in literature, no study investigated the effect of symbiotic and/or Vitamin E supplementation on circulating leptin levels in NAFLD patients. Our results showed that combination of symbiotic and Vitamin E supplementation reduced leptin levels significantly. However, in just one study in NAFLD patients, ursodeoxycholic acid with or without Vitamin E did not affect leptin levels or BMI, despite an improvement in hepatic steatosis.[35] The mechanism by which symbiotics act is through suppressing TLR-related responses by altering the intestinal flora,[36] reducing inflammation, and improving the liver in animals and humans with fatty livers.[9] On the other hand, the exact mechanisms by which Vitamin E supplementation might affect leptin levels are unknown. Vitamin E as an antioxidant may have favorable effects against leptin action on Kupffer cells.[37]

Abnormalities in lipid and lipoprotein metabolism along with chronic inflammation as a result of endotoxemia are considered to be the central pathway for the development of several obesity-related comorbidities such as NAFLD and CVD.[38] Moreover, the inability of the liver to regulate the changes in lipogenesis accompanied by insulin and glucose role in adipogenesis are major causes of fat accumulation in NAFLD patients.[39] In our study, symbiotic and Vitamin E supplementation simultaneously decreased TC, TG, and LDL-C levels significantly, but the results of this study did not show any significant increase in HDL-C levels after intervention. Furthermore, apo B100/A-1, which has a strong relationship with NAFLD, significantly decreased more in symbiotic group. To our knowledge, up to now, no study has examined the effects of symbiotic plus Vitamin E on lipid profile in NAFLD patients. Besides, among studies that assessed the effects of probiotic or symbiotic supplementation, none of them mentioned the lipid profile in NAFLD patients.[9,23] Meanwhile, no study investigated the positive effects of Vitamin E supplementation on lipid profile in these patients.[25] Lipid-lowering properties of symbiotic might be explained by other studies that were conducted in diabetic and obese patients. In these studies, VSL#3 along with prebiotic supplementation induced changes in the microbiota that was associated with an increase in the levels of short-chain fatty acids. Furthermore, probiotic bacteria can remove or assimilate cholesterol and can hydrolyze conjugated bile acids, and so excrete them faster.[40]

IR, the physiopathological key to metabolic syndrome and to its clinically related diseases, is independently associated with NAFLD and its severity;[3,4] however, no study has investigated the effects of symbiotic in combination with Vitamin E supplementation on IR. The results of our study showed a significant reduction in FBS and insulin concentrations but not HOMA-IR. These results agree with the findings of Eslamparast *et al*. that found a significant reduction in FBS and serum insulin levels following symbiotic supplementation.[23] The present findings seem to be consistent with another research which found a significant reduction in serum FBS and serum insulin levels after treatment with *B. Longum* and fructooligosaccharide.[10]

In a study by Yakaryilmaz *et al*., nine patients with biopsy-proven NASH were given oral Vitamin E (800 mg) daily for 24 weeks. At the end of the 6 months, fasting insulin improved, but serum TC, TG, and FBS levels remained unchanged.[41] 800 IU Vitamin E supplementation in another study had no effect on IR. The possible reasons for this discrepancy might be explained by high dose of Vitamin E supplementation and duration of interventions.

Circulating LPS are present at higher concentrations in the blood of patients with type 2 diabetes mellitus or IR and correlate with insulin, glucose concentrations, and HOMA-IR.[42] Increase insulin hypersecretion along with SIBO and increased intestinal permeability; accelerate liver fat accumulation, which lead to NAFLD. Furthermore, gut microbiota has been shown to affect fat storage and energy harvesting, playing an important role in the development of IR.[43] The mechanisms by which symbiotics could improve IR and lipid profile are thought to be by modulation of the intestinal microflora composition, reduction of endotoxemia, increases in fecal pH, suppression of inflammation and reduction in the production and absorption of intestinal toxins.[44] It was well established that Vitamin E reducing IR mechanisms are through suppressing oxidative stress and peroxisome proliferator-activated receptor-alpha expression.[41]

The strengths of our study include its randomized, double-blind, placebo-controlled design, combination of symbiotic and Vitamin E supplementation, and the strong compliance and retention of patients to the experimental program.

The main limitation of this survey is its relatively small sample size and the measurement time which reduced our power to detect differences among subgroups. In addition, in our study, we had to convince the participants to be merely strict on consuming not probiotic products during the study although we could unravel the problem by regular weekly phone calls.

Another limitation of this study is that the diagnosis of liver disease was not confirmed by liver biopsy as it is difficult to perform due to ethical reasons; hence, further studies, which take these variables into account, will need to be undertaken. Compelling of evidence has indicated that bacterial count and using fecal sample provide a better estimate of intervention with probiotics. In subsequent study, we will make every effort to collect fecal samples to evaluate changes in bacterial species and abundance in particular, among NAFLD patients.

CONCLUSION

Significant findings emerged from this study are that 400 IU Vitamin E in combination with symbiotic supplements can decrease the liver enzymes, serum insulin, FBS, and leptin levels along with some lipid profiles in NAFLD patients.

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Conflicts of interest

There are no conflicts of interest.

AUTHORS' CONTRIBUTIONS

- GE carried out the study design and coordinated the study, participated in most of the experiments
- RKM prepared the manuscript, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work
- SA provided assistance in the sample collection and participated in most of the experiments.
- MZ carried out the study design and coordinated the study, participated in most of the experiments and in manuscript preparation
- AFH provided assistance in the statistical analysis
- SSSAS provided assistance in the experiment
- FS carried out the study design and coordinated the study, participated in most of the experiments, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work.

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