

Synbiotic supplementation in nonalcoholic fatty liver disease: a randomized, double-blind, placebo-controlled pilot study^{1–3}

Tannaz Eslamparast, Hossein Poustchi, Farhad Zamani, Maryam Sharafkhah, Reza Malekzadeh, and Azita Hekmatdoost

ABSTRACT

Background: Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world. Oral administration of synbiotic has been proposed as an effective treatment of NAFLD because of its modulating effect on the gut flora, which can influence the gut-liver axis.

Objective: The objective was to evaluate the effects of supplementation with synbiotic on hepatic fibrosis, liver enzymes, and inflammatory markers in patients with NAFLD.

Design: In a randomized, double-blind, placebo-controlled clinical trial conducted as a pilot study, 52 patients with NAFLD were supplemented twice daily for 28 wk with either a synbiotic or a placebo capsule. Both groups were advised to follow an energy-balanced diet and physical activity recommendations.

Results: At the end of the study, the alanine aminotransferase (ALT) concentration decreased in both groups; this reduction was significantly greater in the synbiotic group. At the end of the study, the following significant differences [means (95% CIs)] were seen between the synbiotic and placebo groups, respectively: ALT [−25.1 (−26.2, −24) compared with −7.29 (−9.5, −5.1) IU/L; $P < 0.001$], aspartate aminotransferase [−31.33 (−32.1, −30.5) compared with −7.94 (−11.1, −4.8) IU/L; $P < 0.001$], γ -glutamyltransferase [−15.08 (−15.5, −14.7) compared with −5.21 (−6.6, −3.9) IU/L; $P < 0.001$], high-sensitivity C-reactive protein [−2.3 (−3, −1.5) compared with −1.04 (−1.5, −0.6) mmol/L; $P < 0.05$], tumor necrosis factor- α [−1.4 (−1.7, −1.1) compared with −0.59 (−0.8, −0.3) mmol/L; $P < 0.001$], total nuclear factor κ -B p65 [−0.016 (−0.022, −0.011) compared with 0.001 (−0.004, −0.007) mmol/L; $P < 0.001$], and fibrosis score as determined by transient elastography [−2.98 (−3.6, −2.37) compared with −0.77 (−1.32, −0.22) kPa; $P < 0.001$].

Conclusions: Synbiotic supplementation in addition to lifestyle modification is superior to lifestyle modification alone for the treatment of NAFLD, at least partially through attenuation of inflammatory markers in the body. Whether these effects will be sustained with longer treatment durations remains to be determined. This trial was registered at clinicaltrials.gov as NCT01791959. *Am J Clin Nutr* 2014;99:535–42.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD)⁴ is the most common chronic liver disease in the world and may lead to nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (1). No treatment has yet been approved for NAFLD, and the only recognized management strategies include lifestyle modifications (2).

The concept that gut microbiota, including the billions of bacteria resident within the human gastrointestinal tract, can be involved in the pathogenesis of liver disorders has been proposed. The gut consists of a complex of microorganism species, the concentration and type of which are mostly influenced by host genotype and nutrient availability (3). It is known that the liver is susceptible to the exposure of intestine-derived bacterial products because of a close anatomic and functional connection between the intestinal lumen and the liver through the portal system (4, 5). The gut-liver axis is an important pathway in the development of NAFLD, which is associated with small intestinal bacterial overgrowth and increased intestinal permeability (6–9). The contribution of microflora in the progression of NAFLD is mainly based on increased hepatic oxidative stress due to the increased production of ethanol, and lipopolysaccharides in the intestinal lumen, and subsequent release of inflammatory cytokines in some inflammatory cells (5, 9). Of these inflammatory cytokines, TNF- α appears to play a crucial role in both insulin

¹ From the Department of Clinical Nutrition and Dietetics, Faculty of Nutrition and Food Technology, National Nutrition and Food Technology, Research Institute Shahid Beheshti University of Medical Science, Tehran, Iran (TE and AH); the Liver and Pancreatobiliary Diseases Research Center, Digestive Diseases Research Institute, Tehran University of Medical Sciences, Tehran, Iran (TE, HP, MS, and RM); and the Gastroenterology and Liver Disease Research Center, Firoozgar Hospital, Tehran University of Medical Sciences, Tehran, Iran (FZ).

² Supported by the National Nutrition and Food Technology Research Institute of the Shahid Beheshti University and the Digestive Disease Research Institute of the Shariati Hospital. Protexin Company, UK, provided the synbiotic supplements, and Nikan Teb Co provided the FibroScan machine.

³ Address correspondence to A Hekmatdoost, Department of Clinical Nutrition and Dietetics, Faculty of Nutrition and Food Technology, National Nutrition and Food Technology Research Institute Shahid Beheshti University of Medical Science, 7 No, West Arghavan Street, Farahzadi Boulevard, PO Box 19395-4741, Tehran, Iran 1981619573. E-mail: a_hekmat2000@yahoo.com; and H Poustchi, Liver and Pancreatobiliary Diseases Research Center, Digestive Diseases Research Institute, Tehran University of Medical Sciences, Shariati Hospital, PO Box 14117-13135, Tehran, IR Iran. E-mail: h.poustchi@gmail.com.

⁴ Abbreviations used: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FBS, fasting blood sugar; GGT, γ -glutamyltransferase; MET, metabolic equivalent of task; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NF- κ B, nuclear factor κ -B; PBMC, peripheral blood mononuclear cell; WHR, waist-to-hip ratio.

Received June 19, 2013. Accepted for publication December 12, 2013.

First published online January 8, 2014; doi: 10.3945/ajcn.113.068890.

resistance and hepatic inflammatory cell recruitment in NAFLD/NASH (10–12).

Probiotics are live microorganisms that are beneficial to human health when ingested (13). On the basis of NAFLD characteristics, including steatosis and liver inflammation, followed by fibrosis, and the proven immunomodulatory, antifibrotic and antiinflammatory properties of prebiotics and/or probiotics in animals (14–17) and limited human studies (18, 19), we hypothesized that, in addition to lifestyle modifications (diet and exercise), manipulation of enteric flora by consumption of synbiotic may act as a novel adjunctive therapeutic strategy in patients with NAFLD. To evaluate this hypothesis, a double-blind, randomized, placebo-controlled clinical trial was designed to determine whether supplementation with synbiotic will further improve the efficacy of lifestyle modifications on NAFLD management while addressing some of the mechanisms of action by which synbiotic may function.

SUBJECTS AND METHODS

Recruitment and eligibility screening

Patients were identified and recruited from the Haraz Clinic in Amol, Iran. The diagnosis of NAFLD was made on the basis of the presence of steatosis, on ultrasound examination, associated with a persistently elevated alanine aminotransferase (ALT) concentration of >60 U/L for 6 mo before the study and at the time of randomization. Exclusion criteria included anyone with viral hepatitis, alcohol use, other causes of chronic liver disease, diabetes mellitus, untreated hypothyroidism, clinically or biochemically recognized systemic diseases, psychiatric disorders impairing the patient's ability to provide written informed consent, and pregnancy, lactation, and lack of effective birth control in women of childbearing age. Men and women ≥ 18 y of age were recruited.

Study design

All eligible patients with NAFLD were recruited in March 2012. Interviews and questionnaires were all administered at the Haraz Clinic. Patients signed an informed consent form after a full review of the inclusion and exclusion criteria and an explanation of the risks and benefits of the study, which were approved by the ethics committee of the National Nutrition and Food Technology Research Institute and the Digestive Diseases Research Institute of Tehran University of Medical Sciences. At week 0, patients were randomly assigned to receive either synbiotic supplementation or the identical-appearing placebo capsule (maltodextrin) twice daily for 28 wk, and baseline data were gathered. Follow-up assessments were performed every 7 wk at weeks 7, 14, 21, and 28 after randomization.

According to a multistage, cluster, random-sampling method, 6140 subjects were selected for the NAFLD screening study in Amol, of whom 2460 had a diagnosis of NAFLD based on ultrasound and liver enzyme results (**Figure 1**). Those with the highest fibrosis grades, determined by transient elastography (FibroScan; echosens), who agreed to participate were randomly assigned by age and sex into the synbiotic or placebo group. Randomization lists were computer-generated by a statistician

and given to the interviewer. Subjects, investigators, and staff were blind to the treatment assignment until the end of the study.

At the first visit (week 0), baseline data were gathered and patients were provided with a 7-wk supply of capsules. At each follow-up visit (every 7 wk), patients were given another set of capsules. Each synbiotic capsule (Protexin) contained 200 million of 7 strains of friendly bacteria (*Lactobacillus casei*, *Lactobacillus rhamnosus*, *Streptococcus thermophilus*, *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Lactobacillus bulgaricus*) and prebiotic (fructooligosaccharide) and probiotic cultures [magnesium stearate (source: mineral and vegetable) and a vegetable capsule (hydroxypropyl methyl cellulose)]. Adherence was assessed by capsule counts confirmed at each visit.

Both groups were advised to follow an energy-balanced diet and physical activity recommendations according to the Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults from the NIH and the North American Association for the Study of Obesity (20). The distribution of nutrients in relation to the total caloric value was as follows: $\leq 30\%$ of total energy as fat (10% as SFAs, 15% as MUFAs, and 5% as PUFAs), 15–18% as protein, 52–55% as carbohydrate, <300 mg/d as dietary cholesterol, and 20–30 g fiber/d. Patients were also advised to exercise ≥ 30 min, 3 times/wk.

Clinical, paraclinical, and dietary intake assessments

All patients underwent measurements of weight, height, and waist and hip circumferences. Each individual's BMI was calculated by using the following formula: $\text{BMI} = \text{weight (in kg)} / \text{height (in m)}^2$. Waist-to-hip ratio (WHR) was measured according to WHO recommendation (21).

Biochemical testing was performed on each patient at weeks 0, 7, 14, 21, and 28 after they had fasted for 12 h. All biochemical assessments were performed in the same laboratory by using standard laboratory methods. Total bilirubin and γ -glutamyltransferase (GGT) were measured by enzymatic colorimetric assay (Parsazmoun). ALT, aspartate aminotransferase (AST), and alkaline phosphatase (ALP) concentrations were measured by photometric assay (Reckon). Inflammatory factors were assessed at baseline and at the end of the study. Fasting high-sensitivity C-reactive protein concentrations were measured by ELISA (Diagnostics Biochem Canada Inc). Plasma TNF- α concentrations were measured by using a commercial ELISA kit (KOMA BIOTECH Inc) with a lower sensitivity limit of 10 pg/mL. Nuclear factor κ -B (NF- κ B) p65 was measured in peripheral blood mononuclear cell (PBMC) nuclear extracts by using an ELISA kit (Cell Signaling) according to the manufacturer's protocol.

Fasting glucose concentrations were measured by using the GOD/POD method. Fasting insulin concentrations were measured by ELISA (Mercodia AB). HOMA-IR was used to determine the degree of insulin resistance by using the following formula (22): $\text{HOMA-IR} = [\text{fasting insulin (mU/L)} \times \text{fasting blood glucose (mg/dL)}] / 405$.

After patients were randomly assigned into the 2 groups, liver fibrosis was also assessed by transient elastography at baseline and at the end of the study. Transient elastography was performed by using the same equipment and by the same operator who was also blind to the study randomization and was unaware of the clinical and laboratory results.

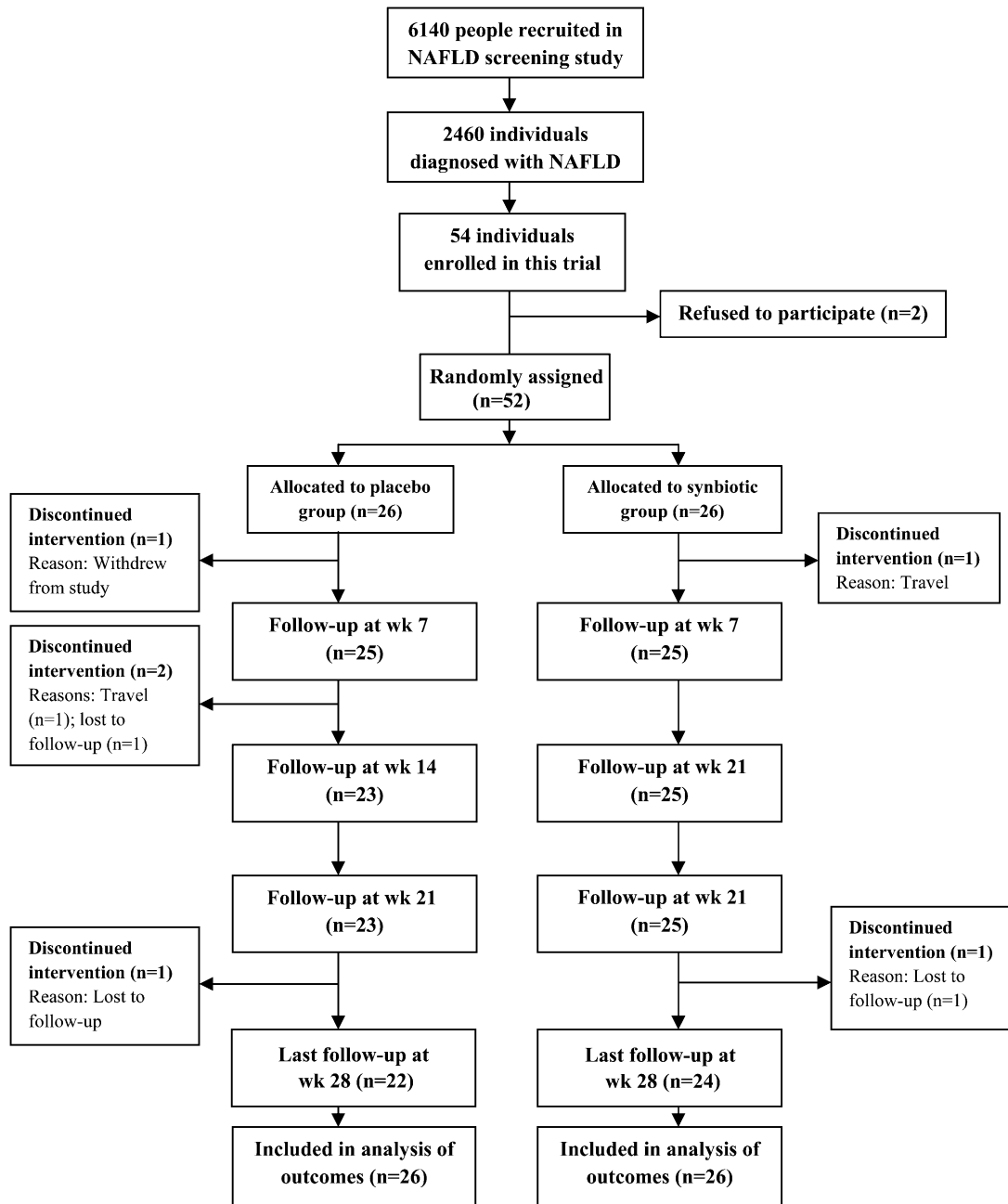


FIGURE 1. Consolidated Standards of Reporting Trials flow diagram of the study participants. NAFLD, nonalcoholic fatty liver disease.

For the assessment of nutrient intakes, patients received food records at weeks 0, 7, 14, 21, and 28 and were instructed to record their daily dietary intake for 3 d, including a weekend day. Dietary intakes were then analyzed by using Nutritionist 4 (First DataBank), incorporating the use of food scales and models to enhance portion size accuracy. National food-composition tables were used as a reference (23). Physical activity was also assessed by using the metabolic equivalent of task (MET) questionnaire (24) at weeks 0, 7, 14, 21, and 28.

Follow-up

Each follow-up visit consisted of data collected through a standardized medical history, 3 food records, anthropometric

measurements, and serum collection, and capsule counts were used to assess adherence to study treatment. An assessment of adverse events that may have occurred was also performed at each visit. The final patient follow-up was in October 2012 and included a transient elastography assay in addition to all of the follow-up assessments mentioned.

Primary and secondary outcomes

The primary outcome measure was a significant reduction in ALT concentration. Secondary outcome measures were transient elastography score, inflammatory factor concentrations in serum and PBMCs, anthropometric variables, and serum concentrations of AST, GGT, total bilirubin, and ALP.

Statistical analysis

Data were analyzed with the use of STATA software (version 11; StataCorp). For all analyses a *P* value <0.05 was considered statistically significant. Continuous and categorical data were presented as means $\pm$ SDs and frequency, respectively. Demographic variables were analyzed by using a chi-square or *t* test, as appropriate.

The data were analyzed according to the intention-to-treat principle. Patients missing laboratory measurements of the primary and secondary outcome measures were imputed. A multiple imputation procedure was used based on Multivariate Imputation by Chained Equations. In the multiple imputation procedure, 5 imputed data sets were generated. The results of the 5 imputed data sets were pooled to obtain data estimates.

Means and 95% CIs for changes from baseline in ALT and AST were compared at 7, 14, 21, and 28 wk by using ANCOVA models for ALT at each time point. ANCOVA models were also used to compare changes from baseline to the end of treatment in transient elastography score, GGT, ALP, and bilirubin. All ANCOVA models were adjusted for the baseline value of each outcome and mean change in BMI, WHR, MET, and energy. To

determine the emergence of any time-related patterns of response to treatment, means and 95% CIs for changes relative to baseline in ALT, AST, and BMI were plotted against time from baseline until the end of treatment.

RESULTS

Characteristics of the patients

Fifty-two patients were included in the study and were randomly assigned into the 2 groups—synbiotic (*n* = 26) or placebo (*n* = 26). Patient screening, enrollment, and retention by treatment group are shown in Figure 1. The baseline clinical and demographic data of the 2 groups were similar with respect to anthropometric data, laboratory data, and liver echogenicity and elasticity (Table 1).

Primary outcome

Eighty-eight percent of patients completed 28 wk of treatment and had end-of-study clinical and laboratory variables obtained as well as transient elastography results. All enrolled patients were included in the analysis of the primary outcome—reduction

TABLE 1
Baseline characteristics at enrollment¹

	Total (<i>n</i> = 52)	Synbiotic group (<i>n</i> = 26)	Placebo group (<i>n</i> = 26)	<i>P</i> value
Age (y)	46.0 $\pm$ 9.2 ²	46.35 $\pm$ 8.8	45.69 $\pm$ 9.5	0.801
Sex (M/F)	25/27	14/12	11/15	0.405
Smoking				1.000
Yes	4	2	2	
No	48	24	24	
Metabolic characteristics				
Height (cm)	162.0 $\pm$ 9.7	163.4 $\pm$ 8.0	160.5 $\pm$ 10.9	0.297
Weight (kg)	83.6 $\pm$ 11.8	85.7 $\pm$ 10.0	81.5 $\pm$ 13.2	0.199
Waist circumference (cm)	102.6 $\pm$ 6.8	102.4 $\pm$ 6.8	102.8 $\pm$ 6.2	0.152
BMI (kg/m ²)	31.7 $\pm$ 2.4	32.1 $\pm$ 2.4	31.3 $\pm$ 2.3	0.247
WHR	—	0.94 $\pm$ 0.1	0.97 $\pm$ 0.1	0.842
MET (h/d)	31.7 $\pm$ 4.2	31.39 $\pm$ 4.1	32.00 $\pm$ 4.2	0.598
Energy (kcal)	2271.0 $\pm$ 452.2	2351.0 $\pm$ 515.1	2190.9 $\pm$ 372.2	0.205
Serum biochemistry tests				
ALT (IU/L)	70.4 $\pm$ 6.6	69.3 $\pm$ 2.3	71.5 $\pm$ 9.1	0.246
AST (IU/L)	67.3 $\pm$ 6.9	66.4 $\pm$ 2.6	68.3 $\pm$ 9.4	0.323
ALP (IU/L)	230.6 $\pm$ 11.2	231.4 $\pm$ 10.4	229.8 $\pm$ 12.1	0.603
GGT (IU/L)	89.2 $\pm$ 2.0	89.5 $\pm$ 1.5	89.0 $\pm$ 2.5	0.370
Bilirubin (mg/dL)	0.96 $\pm$ 0.2	0.96 $\pm$ 0.3	0.95 $\pm$ 0.2	0.831
FBS (mg/dL)	98.2 $\pm$ 18.7	99.6 $\pm$ 24.2	98.9 $\pm$ 21.4	0.217
Insulin (mU/L)	11.1 $\pm$ 4.8	11.2 $\pm$ 3.4	11.1 $\pm$ 4.1	0.967
HOMA-IR	2.7 $\pm$ 1.4	2.8 $\pm$ 1.0	2.7 $\pm$ 1.2	0.877
Inflammatory factors				
hs-CRP (ng/mL)	4.25 $\pm$ 2.7	4.23 $\pm$ 2.7	4.27 $\pm$ 2.8	0.956
TNF- α (pg/mL)	11.79 $\pm$ 3.2	12.39 $\pm$ 3.9	11.20 $\pm$ 2.3	0.181
NF- κ B p65	0.072 $\pm$ 0.02	0.076 $\pm$ 0.01	0.065 $\pm$ 0.02	0.02
Liver histology				
Transient elastography (kPa) ³	8.6 $\pm$ 2.1	9.4 $\pm$ 1.9	7.9 $\pm$ 2.1	0.012
Ultrasound [<i>n</i> (%)]				0.108
Grade 2	37 (71.2)	17 (65.4)	20 (76.9)	
Grade 3	15 (28.8)	9 (34.6)	6 (23.1)	

¹ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FBS, fasting blood sugar; GGT, γ -glutamyltransferase; hs-CRP, high-sensitivity C-reactive protein; MET, metabolic equivalent of task; NF- κ B, nuclear factor κ -B; WHR, waist-to-hip ratio.

²Mean $\pm$ SD (all such values).

³FibroScan (echosens).

in ALT concentration—which decreased significantly in both groups ($P < 0.01$); however, the mean reduction in the synbiotic group was greater than that in the placebo group ($P < 0.001$). The change in ALT concentration at the 21st, and 28th weeks (Table 2) showed a significant difference between those treated with synbiotic or placebo ($P < 0.001$).

Secondary outcomes

We found a significant decrease in the BMI and WHR within both groups; however, no significant differences were observed between the 2 groups in these variables (Figure 2). At the end of the 28-wk treatment period, a significant improvement in several liver variables was seen within and between both groups and before and after the adjustment for MET and energy intake. Compared with the placebo group, patients taking synbiotic capsules had a significantly greater decrease in the following liver enzymes: ALT (69.30 ± 2.3 to 44.20 ± 3.8 in the synbiotic group and 71.46 ± 9.1 to 64.17 ± 11.1 in the placebo group; $P < 0.001$), AST (66.38 ± 2.6 to 35.05 ± 2.7 in the synbiotic group and 68.29 ± 9.41 to 60.34 ± 13.1 in the placebo group; $P < 0.001$), and GGT (89.51 ± 1.5 to 74.42 ± 1.8 in the synbiotic group and 88.99 ± 2.5 to 83.78 ± 3.1 in the placebo group; $P < 0.001$), whereas no significant difference was found within and between the 2 groups in total bilirubin and ALP (Table 2). All of these reductions were significantly different from those at 14 wk.

Fasting blood sugar (FBS) and insulin concentrations also decreased significantly in both groups and between groups before and after adjustments. The reductions in insulin resistance variables of the synbiotic group compared with the placebo group were as follows: FBS decreased as much as -7.96 compared with -2.82 mg/dL ($P < 0.001$), -2.02 compared with -0.92 mU/L ($P < 0.05$), and HOMA-IR -0.68 compared with -0.39 ($P < 0.001$) (Table 2).

At the end of the treatment, the transient elastography results also showed a significant improvement within and between both groups; the mean reduction in the fibrosis score in the synbiotic group was significantly greater than that in the placebo group (9.36 ± 1.9 to 6.38 ± 1.5 in the synbiotic group compared with 7.92 ± 2.1 to 7.16 ± 2.0 in placebo group; $P < 0.001$). In the synbiotic group, 95% of patients showed some improvement in their fibrosis score, whereas the scores of 5% remained unchanged. Of those with improvements, 8% had a 1-level reduction in their fibrosis score, whereas 36% and 56% had 2- and 3-level reductions, respectively. Fibrosis scores also decreased in the placebo group, but less than in the synbiotic group. Only 36% of the patients in this group had one or more levels of reduction in their fibrosis scores.

All of the inflammatory markers decreased after treatment in both groups, although the mean decrease in the synbiotic group was greater than in the placebo group (Table 3). None of the patients completing the study had any serious adverse events, which indicated tolerance to the treatment. Two minor adverse events were reported; one patient complained of moderate headaches and one of abdominal pain in the synbiotic and placebo groups, respectively, both of which were resolved without recurrence.

DISCUSSION

To our knowledge, this was the first randomized, double-blind clinical trial that evaluated the effects of a synbiotic supplement on NAFLD characteristics and addressed some of its mechanisms of action. Our results have shown that coadministration of synbiotic improves the efficacy of lifestyle modifications in patients with NAFLD, at least partially through the inhibition of NF- κ B activation and the reduction of TNF- α production.

Animal studies have shown that translocation of bacterial products, such as lipopolysaccharides, from the intestinal lumen

TABLE 2
Mean changes (95% CIs) from baseline in ALT, AST, GGT, and HOMA-IR by treatment group¹

Change from baseline	Synbiotic group (n = 26)	Placebo group (n = 26)	P value ²
ALT concentration			
Week 7	-8.0 (-10.7, -5.3)	-4.7 (-6.4, -3.1)	0.123
Week 14	-11.3 (-13.4, -9.1)	-4.4 (-5.5, -3.4)	0.02
Week 21	-15.3 (-17.6, -13.1)	-2.5 (-4.4, -0.5)	<0.001
Week 28	-25.1 (-26.2, -24.0)	-7.3 (-9.5, -5.1)	<0.001
AST concentration			
Week 7	-14.4 (-17.3, -11.5)	-4.9 (-6.6, -3.2)	0.01
Week 14	-19.1 (-21.6, -16.6)	-7.7 (-11.6, -3.8)	<0.001
Week 21	-22.5 (-24.8, -20.2)	-5.0 (-8.1, -1.8)	<0.001
Week 28	-31.3 (-32.1, -30.5)	-7.9 (-11.1, -4.8)	<0.001
HOMA-IR			
Week 7	-0.23 (-0.3, -0.2)	-0.11 (-0.2, -0.02)	0.033
Week 14	-0.35 (-0.4, -0.2)	-0.12 (-0.2, -0.04)	<0.001
Week 21	-0.43 (-0.5, -0.3)	-0.13 (-0.2, -0.04)	<0.001
Week 28	-0.68 (-0.8, -0.5)	-0.39 (-0.5, -0.3)	<0.001
GGT concentration			
Week 14	-7.54 (-7.7, -7.3)	-2.61 (-3.3, -1.9)	<0.001
Week 28	-15.08 (-15.5, -14.7)	-5.21 (-6.6, -3.9)	<0.001

¹ ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ -glutamyltransferase.

² Based on an ANCOVA model that regressed changes from baseline on treatment group, baseline value of the outcome, and mean change in BMI, waist-to-hip ratio, metabolic equivalent tasks, and energy.

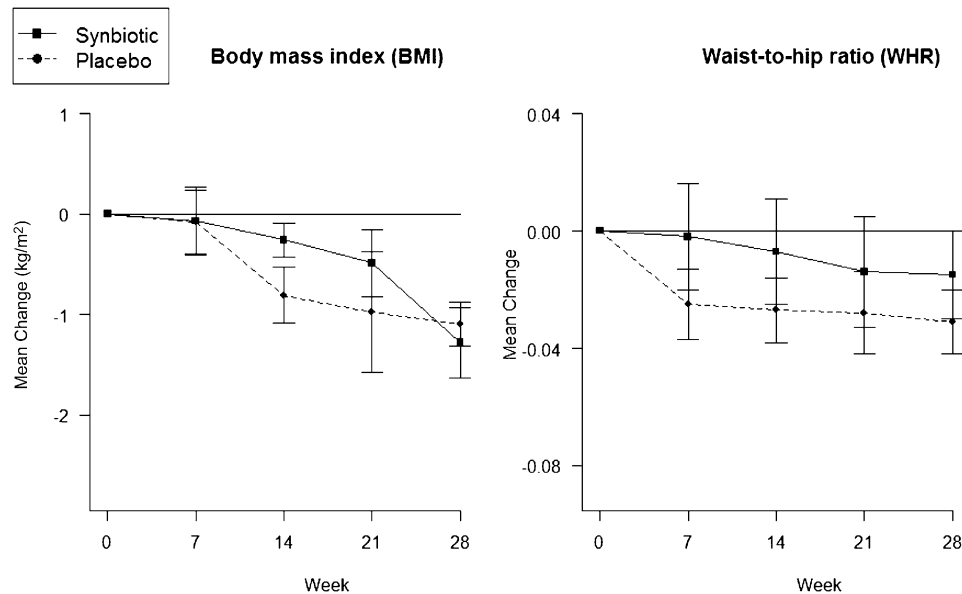


FIGURE 2. Changes in BMI and WHR during treatment and follow-up. The number of patients at each visit and within each treatment group was 26. Error bars indicate 95% CIs. BMI and WHR decreased significantly in both groups ($P < 0.05$); no significant differences were observed between the 2 groups. WHR, waist-to-hip ratio.

to the mesenteric lymphatic circulation activates the immune system, which induces the regional and systemic production of proinflammatory cytokines and enhances the production of free radical species in the abdominal area, which might contribute to the evolution of NAFLD and steatohepatitis (25). Of these inflammatory cytokines, TNF- α appears to play a critical role in both insulin resistance and hepatic inflammatory cell recruitment in NAFLD/NASH (10–12). The production of TNF- α occurs as a result of NF- κ B activation, which is a master regulator of inflammation (10, 26).

Most of the previous animal studies that evaluated the effect of probiotics on NAFLD have shown that probiotics can ameliorate NAFLD characteristics and inflammatory factors such as NF- κ B activation and TNF- α secretion (15, 17); however, the findings regarding the expression of TNF- α in hepatic tissue are controversial (14, 16). Li et al (16) investigated the effect of VSL#3 on an experimental model of high-fat diet-induced fatty liver and reported that VSL#3 improved liver histologic results, reduced the content of hepatic total fatty acids, and decreased serum ALT concentrations similarly to the anti-TNF- α antibodies. These effects were associated with a reduction in Jun N-terminal kinase, a TNF-regulated stress kinase that promotes hepatic insulin resistance, and NF- κ B activity, fatty acid β -oxidation, and mitochondrial uncoupling protein-2 expression, all being markers and factors characterizing insulin resistance;

however they did not observe a reduction in hepatic TNF- α gene expression. They concluded that because VSL#3 therapy reduced hepatic activity of Jun N-terminal kinase, it is conceivable that it inhibited the production of TNF- α by some extrahepatic sources or operated at posttranscriptional levels to block the actions of TNF- α within the liver. Our data support their conclusion because we have observed that synbiotic supplementation reduced NF- κ B activation in PBMCs and total TNF- α in serum, which indicates that probiotics can act as immunomodulatory agents in some extrahepatic organs.

Insulin resistance is one of the characteristics of NAFLD and of the metabolic syndrome; however, only a small number of studies have investigated the effects of synbiotic therapy on insulin resistance. The results of our study showed a significant reduction in FBS and insulin concentrations and an improvement in HOMA-IR. Malaguarnera et al (19) also showed a significant reduction in serum FBS, insulin, and HOMA-IR after treatment with *B. longum* plus fructooligosaccharide, which is consistent with the results of our study. These results are also in line with those of other previous studies (17, 27). It has been found that lipopolysaccharides are present at higher concentrations in the blood of subjects with type 2 diabetes mellitus or insulin resistance than in healthy subjects. Circulating lipopolysaccharides have also been shown to correlate with insulin and glucose concentrations and with HOMA-IR (28, 29). In response to

TABLE 3
Mean changes (95% CIs) from baseline to end of treatment in inflammatory factors by treatment group¹

Change from baseline to end of treatment	Synbiotic group (n = 26)	Placebo group (n = 26)	P value ²
hs-CRP (ng/mL)	-2.30 (-3.0, -1.5)	-1.04 (-1.5, -0.6)	<0.001
TNF- α (pg/mL)	-1.40 (-1.7, -1.1)	-0.59 (-0.8, -0.3)	<0.001
NF- κ B p65	-0.016 (-0.022, -0.011)	0.001 (-0.004, -0.007)	0.006

¹ hs-CRP, high-sensitivity C-reactive protein; NF- κ B, nuclear factor κ -B.
² Based on an ANCOVA model that regressed changes from baseline on treatment group, baseline value of the outcome, and mean change in BMI, waist-to-hip ratio, metabolic equivalent tasks, and energy.

systemic insulin resistance, pancreatic β cells increase insulin hypersecretion and accelerate liver fat accumulation, which leads to NAFLD. Synbiotic could improve insulin resistance through mechanisms such as modifications to the gut flora, reductions in endotoxin concentrations, increases in fecal pH, and reductions in the production and absorption of intestinal toxins (7, 25).

One limitation of our study was the lack of liver biopsy results from which to derive a pathology score of disease; however, we did use transient elastography, which provides a quantitative, noninvasive evaluation of NAFLD by measuring hepatic fibrosis (30, 31). This technique has proven to be a reliable, noninvasive method for identifying patients with significant hepatic fibrosis. It is readily reproducible, and its score has low inter- and intra-observer variability (30, 31). In addition, because patients were evaluated with FibroScan after being randomly assigned, the transient elastography scores of the 2 groups were different at baseline: the placebo group had less fibrosis (average score: 7.92 ± 2.1) than did the synbiotic group (average score: 9.36 ± 1.9). This was an unintentional occurrence, which definitely contributed to the significant difference seen in the fibrosis scores of the 2 groups. Regardless of this, however, the transient elastography scores still decreased significantly—by almost 3 points (from 9.36 ± 1.9 to 6.38 ± 1.5)—in the synbiotic group before and after treatment, which indicated an influence of supplementation on liver fibrosis. Another limitation of this study was that we did not evaluate the gut microbiome, which could have indicated the effects of synbiotic consumption on gut microflora and confirmed our suggested mechanism of action.

The most important strengths of the current study were the relatively long evaluation period of NF- κ B activity in PBMCs, the stratified blocked randomization design, and the inclusion of patients with newly diagnosed NAFLD who had not yet received treatment; all of these strengths are unique in comparison with the few other clinical trials that have evaluated the effects of probiotics alone or in combination with prebiotics on NAFLD (18, 19).

In conclusion, this randomized, double-blind, placebo-controlled trial found some evidence that synbiotic supplementation in addition to lifestyle modification is superior to lifestyle modification alone for the treatment of NAFLD, at least partially through attenuation of inflammatory markers in the body. This effect was seen beginning at week 14, and this trend was sustained until the end of the study. Whether these effects will be sustained with longer treatment durations remains to be determined.

We are indebted to the 17-Shahrivar Hospital in Amol for providing the equipment used in this study and to M. Fallah, the laboratory specialist who performed all laboratory procedures.

The authors' responsibilities were as follows—AH: had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis; AH, HP, FZ, RM, and TE: conceived and designed the study and provided administrative, technical, or material support; MS, TE, and AH: analyzed and interpreted the data; TE and AH: drafted the manuscript; AH, HP, FZ, RM, TE, and MS: critically revised the manuscript for important intellectual content; MS and TE: conducted the statistical analysis; and AH, HP, FZ, and RM: obtained funding and supervised the study. None of the authors had a conflict of interest.

REFERENCES

- Adams LA, Sanderson S, Lindor KD, Angulo P. The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J Hepatol* 2005;42(1):132–8.
- Promrat K, Kleiner DE, Niemeier HM, Jackvony E, Kearns M, Wands J, Fava J, Wing R. Randomized controlled trial testing the effects of weight loss on nonalcoholic steatohepatitis. *Hepatology* 2010;51:121–9.
- Tandon P, Garcia-Tsao G. Bacterial infections, sepsis, and multiorgan failure in cirrhosis. *Semin Liver Dis* 2008;28:26–42.
- Yang L, Seki E. Toll-like receptors in liver fibrosis: cellular crosstalk and mechanisms. *Front Physiol* 2012;3:138.
- Gratz SW, Mykkanen H, El-Nezami HS. Probiotics and gut health: a special focus on liver diseases. *World J Gastroenterol* 2010;16:403–10.
- Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002;346:1221–31.
- Compare D, Coccoli P, Rocco A, Nardone OM, De Maria S, Carteni M, Nardone G. Gut–liver axis: the impact of gut microbiota on non alcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis* 2012;22:471–6.
- Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999;116:1413–9.
- Miele L, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, Masciana R, Forgione A, Gabrieli ML, Perotti G. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. *Hepatology* 2009;49:1877–87.
- Larter CZ, Farrell GC. Insulin resistance, adiponectin, cytokines in NASH: Which is the best target to treat? *J Hepatol* 2006;44(2):253–61.
- Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology* 2006;43(S1):S99–S112.
- Tilg H, Hotamisligil GS. Nonalcoholic fatty liver disease: cytokine-adipokine interplay and regulation of insulin resistance. *J Gastroenterol* 2006;131(3):934–45.
- Rijkers GT, de Vos WM, Brummer R-J, Morelli L, Corthier G, Marteau P. Health benefits and health claims of probiotics: bridging science and marketing. *Br J Nutr* 2011;106(9):1291–6.
- Velayudham A, Dolganiuc A, Ellis M, Petrasek J, Kodys K, Mandrekar P, Szabo G. VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced nonalcoholic steatohepatitis model in mice. *Hepatology* 2009;49(3):989–97.
- Nardone G, Compare D, Liguori E, Di Mauro V, Rocca A, Barone M, Napoli A, Lapi D, Iovene MR, Colantuoni A. Protective effects of *Lactobacillus paracasei* F19 in a rat model of oxidative and metabolic hepatic injury. *Am J Physiol Gastrointest Liver Physiol* 2010;299(3):G669–G76.
- Li Z, Yang S, Lin H, Huang J, Watkins PA, Moser AB, Desimone C, Song XY, Diehl AM. Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. *Hepatology* 2003;37(2):343–50.
- Ma X, Hua J, Li Z. Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. *J Hepatol* 2008;49(5):821–30.
- Loguercio C, Federico A, Tuccillo C, Terracciano F, D'Auria MV, De Simone C, Del Vecchio Blanco C. Beneficial effects of a probiotic VSL#3 on parameters of liver dysfunction in chronic liver diseases. *J Clin Gastroenterol* 2005;39(6):540–3.
- Malaguarnera M, Vacante M, Antic T, Giordano M, Chisari G, Acquaviva R, Mastrojeni S, Malaguarnera G, Mistretta A, Li Volti G, et al. *Bifidobacterium longum* with fructo-oligosaccharides in patients with non alcoholic steatohepatitis. *Digestive diseases and sciences* 2011: 1–9.
- National Heart, Lung, and Blood Institute. The Practical Guide: identification, evaluation, and treatment of overweight and obesity in adults. Bethesda, MD: National Institutes of Health, National Heart, Lung, and Blood Institute, NHLBI Obesity Education Initiative, North American Association for the Study of Obesity, 2000.
- Organization WH. STEPwise approach to surveillance (STEPS). Geneva, Switzerland: World Health Organization, 2012.
- Matthews D, Hosker J, Rudenski A, Naylor B, Treacher D, Turner R. Homeostasis model assessment: insulin resistance and β -cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;28(7):412–9.
- Ghaffarpour M, Houshiar-Rad A, Kianfar H. The manual for household measures, cooking yields factors and edible portion of foods. Tehran, Iran: Nashre Olume Keshavarzy 1999.
- Ainsworth BE, Haskell WL, Whitt MC, Irwin ML, Swartz AM, Strath SJ, O'Brien WL, Bassett DR, Schmitz KH, Emplaincourt

- PO. Compendium of physical activities: an update of activity codes and MET intensities. *Med Sci Sports Exerc* 2000;32(suppl): 498–504.
25. Iacono A, Raso GM, Canani RB, Calignano A, Meli R. Probiotics as an emerging therapeutic strategy to treat NAFLD: focus on molecular and biochemical mechanisms. *J Nutr Biochem* 2011;22(8):699–711.
26. dela Peña A, Leclercq I, Field J, George J, Jones B, Farrell G. NF- κ B Activation, Rather Than TNF, Mediates Hepatic Inflammation in a Murine Dietary Model of Steatohepatitis. *Gastroenterology* 2005;129(5):1663–74.
27. Yadav H, Jain S, Sinha PR. Antidiabetic effect of probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* in high fructose fed rats. *Nutrition* 2007;23:62–8.
28. Creely SJ, McTernan PG, Kusminski CM, Da Silva NF, Khanolkar M, Evans M, Harte AL, Kumar S. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am J Physiol Endocrinol Metab* 2007;292:E740–7.
29. Al-Attas OS, Al-Daghri NM, Al-Rubeaan K, Da Silva NF, Sabico SL, Kumar S, McTernan PG, Harte AL. Changes in endotoxin levels in T2DM subjects on anti-diabetic therapies. *Cardiovasc Diabetol* 2009; 8:20.
30. Malekzadeh R, Poustchi H. Fibroscan for assessing liver fibrosis: an acceptable alternative for liver biopsy: Fibroscan: an acceptable alternative for liver biopsy. *Hepat Mon* 2011;11:157–8.
31. Abenavoli L, Beaugrand M. Transient elastography in non-alcoholic fatty liver disease. *Ann Hepatol* 2012;11:172–8.