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ORIGINAL ARTICLE

Beneficial effects of probiotic combination with omega-3 fatty acids in NAFLD: a randomized clinical study

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ABSTRACT

BACKGROUND: The manipulation of gut microbiota via administration of probiotics has been proposed as a potential strategy for the treatment of non-alcoholic fatty liver disease (NAFLD). Hence, we performed a double-blind single center randomized placebo-controlled trial (RCT) to evaluate the efficacy of coadministration of probiotics with omega-3 *vs*. placebo in type-2 diabetic patients with NAFLD.

METHODS: A total of 48 patients met the criteria for inclusion. They were randomly assigned to receive "Symbiter Omega" combination of probiotic biomass supplemented with flax and wheat germ oil (250 mg of each, concentration of omega-3 fatty acids 1-5%) or placebo for 8-weeks. The primary main outcomes were the change in fatty liver index (FLI) and liver stiffness (LS) measured by Shear Wave Elastography (SWE). Secondary outcomes were the changes in transaminases level, serum lipids and cytokines levels.

RESULTS: In probiotic-omega group, FLI significantly decreased from 83.53±2.60 to 76.26±2.96 (P<0.001) while no significant changes were observed in the placebo group (82.86±2.45 to 81.09±2.84; P=0.156). Changes of LS in both groups were insignificant. Analysis of secondary outcomes showed that the coadministration of probiotics with omega-3 lead to significant reduction of serum gamma-glutamyl transpeptidase, triglycerides, and total cholesterol. Chronic systemic inflammatory markers after intervention decrease significantly only in Symbiter Omega group: IL-1β (P=0.029), TNF-α (P<0.001), IL-8 (P=0.029), IL-6 (P=0.003), and INF-γ (P=0.016).

CONCLUSIONS: Coadministration of a live multi-strain probiotic mixture with omega-3 fatty acids once daily for 8 weeks to patients with NAFLD can reduce liver fat, improve serum lipids, metabolic profile, and reduce chronic systemic inflammatory state.

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KEY WORDS: Probiotics - Lactobacillus - Bifidobacterium - Fatty acids, omega-3 - Propionibacterium - Non-alcoholic fatty liver disease.

In the last decades, the epidemiology of chronic liver diseases is changing due to the decreasing rate of viral hepatitis and the increasing new epidemic of a wide spectrum of metabolic disorders like steatosis, non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH).[1, 2]

NAFLD is characterized by the accumulation of lipids within the hepatocytes exceeding 5% of liver weight in the absence of excessive alcohol intake or other causes of liver diseases, and ranges from simple steatosis to NASH that can have different degrees of fibrosis and progress to liver cirrhosis and hepatocellular carcinoma (HCC).[3-5] NAFLD is related to insulin resistance which appears to be a key mediator in the initiation and progression of disease, mainly through adverse changes in glucose, fatty acid (FA) and lipoprotein metabolism.[6-8]

Currently there are no drugs approved for the treatment of NAFLD. However, despite the abundance of clinical trials, several pharmacologic treatments for NAFLD/NASH are proposed, such as those originally developed for insulin-resistant states and weight loss, metformin, and thiazolidinediones,[9-12] lipid-lowering drugs,[13] antioxidants,[11, 12] and antitumor necrosis factor (TNF)-α agents.[14]

Recently, the manipulation of gut microbiota via administration of probiotics has been proposed as a potential strategy for the NAFLD treatment.[15, 16] Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[17] The data from animal studies suggested that probiotics and prebiotics have been shown to change the microbiota components and to influence food digestion, regulating appetite and body weight.[18] Their beneficial effects can influence glucose and fat metabolism, improve insulin sensitivity, and reduce chronic systemic inflammation.[19] Our choice of probiotics for the present study is based on previous comparative experimental studies of different probiotic strains intended for treatment and prevention of NAFLD and obesity.[20, 21] Administration to animals of poly-probiotic mixtures containing both alive and lyophilized strains, led to significant decreases of total and visceral adipose tissue weight, steatosis and liver lobular inflammation, and to increased insulin sensitivity in animals with experimental obesity.[20, 21] Furthermore, more pronounced changes were seen following administration of a probiotic mixture containing mostly live strains compared to lyophilized ones.

Nutraceuticals, in particular short-chain and polyunsaturated fatty acids (PUFA) belonging to omega-3 and omega-6, sorbents, are substrates-pharmaceuticals which are able to realize immunomodulating, cytoprotective, and antioxidant functions, to take part in metabolic processes, and to deliver energetic and plastic materials to epitheliocytes. The combined administration of nutraceuticals and probiotics allows to simultaneously improve mucosal cytoprotection and to restore the symbiosis between intestine and physiological microflora.[22]

The background of the present randomized clinical trial (RCT) was reported in a previous study, in which we demonstrated in an animal model that a live probiotic mixture in combination with omega-3 fatty acids (FA) was more effective in obesity and NAFLD prevention than probiotic alone.[23, 24] Supplementation of probiotics with omega-3 FA (Symbiter-Omega) led to a significant reduction of steatosis degree, which was accompanied by a significant decrease of triglycerides accumulation in the liver, as compared to the administration of probiotics alone.

In consideration of our preliminary experimental data, the aim of the current study was to perform a double-blind single center RCT to evaluate the efficacy of coadministration of probiotics with omega-3 *vs*. placebo in type-2 diabetic patients with NAFLD.

Materials and methods

The study protocol was approved by local ethics committees of Kyiv City Clinical Endocrinology Center, Ukraine, and was conducted according to the guidelines of the 1975 Declaration of Helsinki. Prior to the study, purpose and methodology of the study were fully explained to the participants by the researchers, and all patients gave written informed consent before any study procedures were initiated.

Study design

In this single-center double-blind, placebo controlled, parallel group study, 48 type-2 diabetes patients were selected. This study was registered at ClinicalTrials.gov under identifier – NCT03528707. They were randomly assigned to receive “Symbiter Omega” or placebo for 8

weeks, administered as a sachet formulation in double-blind treatment. Randomization was done by the study statistician based on a computer-generated list. The groups were homogeneous according to age, sex and diagnostic criteria. The assignment of groups was blind to participants, research staff and outcome assessors moreover, to maintain blind parallel study the statistician was not aware of the allocation of participants to intervention.

The "Symbiter Omega" was supplied by Scientific and Production Company "O.D. Prolisok." It contains combination of flax and wheat germ oil (250 mg of each, concentration of omega-3 fatty acids 1-5%), supplemented with biomass of 14 alive probiotic strains: *Lactobacillus* + *Lactococcus* (6×10^{10} CFU/g), *Bifidobacterium* (1×10^{10}/g), *Propionibacterium* (3×10^{10}/g), *Acetobacter* (1×10^{6}/g) genera. Over 8 weeks of interventional period, the patients received 1 sachet (10 grams) of probiotic-omega and placebo per day. All sachets were identical with similar organoleptic characteristics (*e.g.*, taste and appearance).

The prerandomization period was designed to minimize the effects of dietary changes on metabolic markers. For this purpose, 2 weeks before the study started, after the informed consent was signed, patients were instructed in one-on-one sessions with a dietitian to follow a therapeutic lifestyle-change diet as classified by the National Cholesterol Education Program (NCEP). In addition, participants were instructed to continue with stable antihyperglycemic treatment and received standardized mild physical training for 1 hour per day.

Patients who underwent the study were instructed to take the trial medication as prescribed. Throughout the study, weekly phone follow-up visits were provided for assessment of compliance, adherence to the protocol, as well as the recording of adverse events. The effectiveness of therapy was compared and evaluated separately in the two groups.

Inclusion criteria

Adult participants (age 18-65, Body Mass Index [BMI] ≥25 kg/m^2) diagnosed with NAFLD according to the recommendations of the American Gastroenterology Association (AGA) and American Association for the Study of Liver Disease (AASLD) on the basis of the following parameters: clinical examination, laboratory values of lipid and carbohydrate metabolism, liver enzyme activities (alanine aminotransferase - ALT, aspartate transaminase - AST), ALT/AST ratio, and ultrasonography (US) examination.[6, 25] The diagnosis of fatty liver was based on the results of abdominal US, which was done by trained technicians with Ultima PA (Radmir Co., Kharkiv, Ukraine). Of 4 known criteria (hepato-renal echo contrast, liver brightness, deep attenuation, and vascular blurring), the participants were required to have hepato-renal contrast and liver brightness to be given a diagnosis of NAFLD.[6] Individuals with type-2 diabetes treated with diet and exercise alone or metformin, sulfonylurea (explain this acronym) and insulin will be included, as well as individuals with AST and ALT ≤3x upper limit of normal. Eligible participants must receive antidiabetic drug at stable dose at least 4 weeks prior to the commencement of the study.

Exclusion criteria

Main exclusion criteria included alcohol abuse (>20 g/day — 2 standard drinks — in women, or>30 g/d — 3 standard drinks — in men over a two-year period), chronic viral hepatitis, drug-induced liver disease, Wilson's disease, hereditary deficiency of antitrypsin-1, autoimmune liver diseases, primary biliary cholangitis and idiopathic hemochromatosis; history of decompensated liver disease including ascites, encephalopathy or variceal bleeding; regular use of a probiotic or prebiotic supplement within 3 months prior to enrollment; antibiotic use within 3 months prior to enrollment; uncontrolled cardiovascular or respiratory disease, active malignancy, or chronic infections; use of agents such as vitamin E, omega-3 fatty acids or medications with evidence for effects on NAFLD (pioglitazone, GLP-1 analogues, dipeptidyl peptidase IV inhibitors, ursodeoxycholic acid); and presence of active infection, pregnancy or lactation.

Outcomes assessment

The primary main outcomes were the changes in fatty liver index (FLI) and liver stiffness (LS) measured by Shear Wave Elastography (SWE).

LS was measured by SWE using a multifrequency convex transducer (2-5 MHz) and Ultima PA ultrasound equipment (Radmir, Ukraine). Firstly, in B-mode we estimated the position of liver (the ratio of the edge to the costal arch, the availability of acoustic windows) investigated both lobes of the liver and carried out their anteroposterior size biometrics on inspiration. Even or uneven contour of the liver was assessed, as well as acute or rounded front-bottom corner of the liver. We evaluated the echogenicity (normal, low or high) and echostructure (fine particles - 1-2 mm, medium particles - 3-4 mm, and coarse particles - more than 5 mm). Sound conductivity of the liver parenchyma or opposite US attenuation in the front-rear direction of liver was evaluated by Hamaguchi's B-mode criteria.[26]

SWE was carried out by the standard algorithm for 2D SWE. Especially carefully navigated region of interests (ROI) and SV of 2D SWE by B-mode and removed SWE artifacts. We performed 10 valid measurements of LS in every patient, and a median value was calculated, the result being measured in kPa.

FLI is a validated prediction score for hepatic steatosis severity designed by Bedogni *et al.*.[27] FLI was calculated on the basis of laboratory and anthropometric measures, including triglycerides (TG), gamma glutamyl transferase (GGT), BMI, and waist circumference (WC), by using the following formula:

$$FLI = [e^{0.953*\log e(\text{triglycerides}) + 0.139*BMI + 0.718*\log e(ggt) + 0.053*\text{waist circumference} - 15.745}) / (1 + e^{0.953*\log e(\text{triglycerides}) + 0.139*BMI + 0.718*\log e(ggt) + 0.053*\text{waist circumference} - 15.745})] \times 100$$

Secondary outcomes were the changes in transaminases level, serum lipids and cytokines (TNF-α, IL-1β, IL-6, IL-8, and IFN-γ) levels. All values were determined following a 12-h fasting period, by the hospital clinical laboratory.

Anthropometric data including weight and height were measured to an accuracy of 0.1 kg and 0.5 cm, respectively. BMI was calculated as body weight in kilograms divided by the square of the participant's height in meters (weight/height^2). WC was defined the narrowest diameter between xiphoid process and iliac crest.

Level of alanine (ALT) and aspartate aminotransferase (AST) in serum were determined by the standard biochemical methods. Serum concentrations of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and TG concentrations were measured using the standard enzymatic methods with commercially available kits (BioVendor, Czech Republic). LDL-cholesterol concentration was calculated using the Friedewald equation.[28]

The contents of serum interleukins (TNF-α, IL-1β, IL-6, IL-8, and IFN-γ) were measured by ELISA method using specific mono- and polyclonal antibodies (Sigma) to these proteins. Studied molecules were immobilized in 96-well plates with sorption surface.

Statistical analysis

The SPSS statistical package, version 20.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism, version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) were used for all statistical analyses and a P value less than 0.05 was considered statistically significant. All data in this study were expressed as mean±standard deviation (M±SD) or %. Data distribution was analyzed using the Kolmogorov-Smirnov Normality Test. The baseline characteristics of participants in the 2 groups were compared using independent sample *t*-tests and χ^2 test. The changes in outcomes of the participants after the initiation of therapy and end of the trial were compared by paired sample *t*-tests. Analysis of covariance (ANCOVA) was used to identify any differences between the 2 groups after intervention, adjusting for baseline measurements and confounders (BMI and sex).

Results

A total of 48 patients were randomly divided into two groups receiving either probiotic-omega (N.=26) or placebo (N.=22), respectively. All subjects completed the study and received more than 85% of prescribed sachets, with comparable compliance rate (placebo =86.4% *vs*. probiotics =88.5%, P=0.827). Both probiotics and placebo were well tolerated and generally acceptable by the participants. Patients were satisfied with the organoleptic properties of both. During study period the participants reported only several minor

TABLE I.—*Anthropometric, clinical and laboratory parameters in examined patients (M±SD or %).*

Parameters	Placebo group (N.=22)	Probiotic_omega group (N.=26)	P
Age, years	53.91±11.45	53.92±9.42	0.996
Duration of T2D, years	5.36±2.85	8.00±6.39	0.081
Metformin, % (N.)	68.2 (15)	69.2 (18)	0.999
Sulfonilureas, % (N.)	45.5 (10)	34.6 (9)	0.557
Insulinotherapy, % (N.)	18.2 (4)	34.6 (9)	0.329
FLI	82.86±13.32	83.53±13.29	0.862
LS, kPa	7.2±1.1	7.18±0.95	0.947
BMI, kg/m^2	34.12±6.52	33.18±5.63	0.592
Weight, kg	94.09±15.11	96.95±18.33	0.563
Waist circumference, cm	95.68±6.65	97.80±7.33	0.302
ALT, IU/L	33.85±10.15	34.11±14.64	0.944
AST, IU/L	33.15±9.79	27.74±11.5	0.089
γ-GT, IU/L	51.63±16.11	51.92±20.12	0.957
Triglycerides, mmol/L	2.73±0.94	2.72±0.97	0.972
Total cholesterol, mmol/L	5.9±0.85	6.03±0.76	0.572
HDL-C, mmol/L	1.37±0.26	1.32±0.26	0.483
LDL-C, mmol/L	3.41±0.74	3.48±0.75	0.746
VLDL-C, mmol/L	1.18±0.38	1.23±0.46	0.680
TNF-α, pg/mL	47.61±20.01	49.66±17.94	0.709
IL-1β, pg/mL	43.68±25.59	38.12±15.83	0.363
IL-6, pg/mL	15.08±8.06	14.75±11.30	0.910
IL-8, pg/mL	24.67±8.69	28.06±7.33	0.150
γ-INF	156.54±87.58	178.45±68.83	0.337

adverse events. In the probiotic-omega group, 3 types of adverse events were observed: mild abdominal pain, headache and flatulence. In the placebo group two patients reported nausea. In all cases adverse events reversed spontaneously, had no impact on treatment and did not lead to withdrawn of patients from the study. The prevalence of adverse events was comparable between groups (placebo=9.1% *vs*. probiotics=11.5%, P=0.782).

Table I presents the baseline clinical, anthropometric, and laboratory characteristics of the participants. Participants were treated with oral antidiabetic agents, insulin or their combination. The proportion of participants on insulin-therapy (P=0.329), treated with sulfonylureas (P=0.557) and/or metformin (P=0.999) were comparable between the groups (Table I). In light of latest evidence, some antidiabetic drugs (such us metformin, GLP-1, DPP-4 inhibitors) are known to have pleiotropic effects beyond glucose reduction, including improvement of lipid profiles, bile acids and finally gut microbiota. To avoid impact of these factors we excluded patients treated with incretins. It would therefore be unethical to exclude metformin from their therapy regimen because this drug is an essential therapy for these patients. So, in the present study we randomized equal portions of patients, treated with stable doses of drug at least 4 weeks prior to study start. Moreover, the mean doses of metformin were well balanced and comparable between the groups at baseline.

There were no significant differences between the groups at baseline in terms of age, sex, diabetes duration and treatment or anthropometric measurements. Baseline characteristics of primary and secondary outcomes were evenly distributed across the two groups of enrolled patients (Table I).

Changes of FLI as our main primary outcome are presented in Figure 1. We mentioned slightly significant reduction of FLI from baseline to the eighth week of intervention only in Symbiter-Omega group - from 83.53±13.29 to 76.26±14.97 (P<0.001) (Figure 1A, B). In placebo group our primary endpoint remained static after intervention (82.86±13.32 to 81.09±13.3; P=0.156) (Figure 1A, B). We also observed significant differences in inter-group comparisons between mean changes of FLI expressed in absolute value (Figure 1C) or percentages (Figure

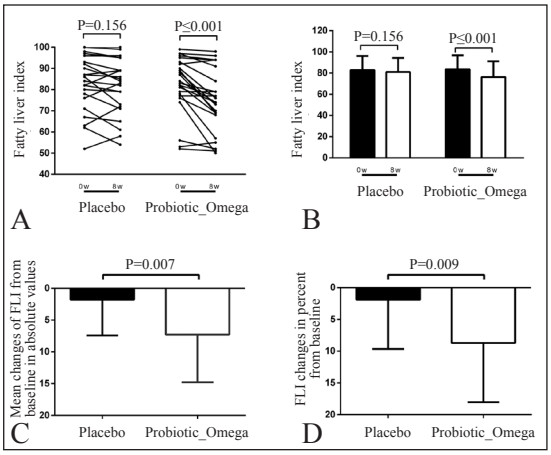

Figure 1.—Primary outcomes analysis with accent on FLI changes. A, B) Intra-group analysis of changes at baseline and after interventon. Data expressed in mean±SEM (A) and individual values at baseline and after 8 weeks of treatment; C, D) analysis of inter-group mean changes of absolute values (C) or percentages (D) from baseline to end of treatment throughout the study. Data expressed as mean±SD. ANCOVA was used to identify any differences between the 2 groups after intervention.

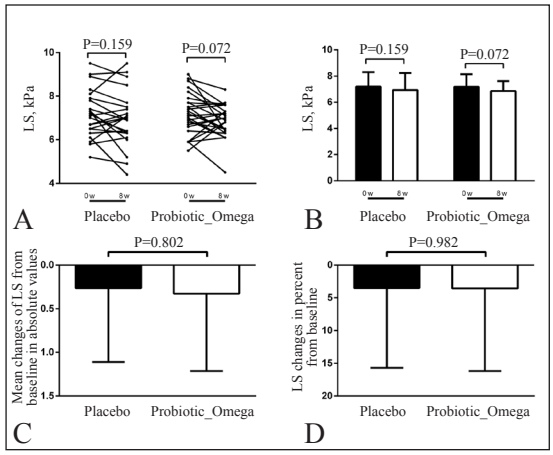

Figure 2.—Primary outcomes analysis with accent on LS changes. A, B) Intra-group analysis of changes at baseline and after interventon. Data expreesed in mean±SEM (A) and individual values at baseline and after 8 weeks of treatment; C, D) analysis of inter-group mean changes of absolute values (C) or percentages (D) from baseline to end of treatment throughout the study. Data expressed as mean±SD. ANCOVA was used to identify any differences between the 2 groups after intervention.

1D) from baseline to end of treatment in ANCOVA analysis.

In respect to our other primary endpoint, the changes of LS measured by SWE in both interventional groups were not significant, but a more pronounced decrease was observed in the Symbiter-Omega group (7.185±0.95 to 6.8 ±0.75; P=0.072) as compared to placebo (7.20±1.1 to 6.94±1.29; P=0.159) (Figure 2A, B). The mean changes of LS from baseline according to ANCOVA analysis between groups were also non-significant: for absolute values — P=0.802 (Figure 2C) — and for percentages from baseline – P=0.982 (Figure 2D).

Analysis of secondary outcomes is presented in Table II. Coadministration of probiotics with omega-3 lead to a significant reduction of GGT serum levels in 14.1% (P<0.001), however in between group comparison using ANCOVA, changes of mean for both absolute values and percentages from baseline were not significant (Figure 3C, Table II). The level of transaminases changed as compared to baseline after Symbiter-Omega, albeit not significantly: AST —1.3% (P=0.310) and ALT —3.2% (P=0.204) (Figure 3A, B, Table II).

Markers of chronic systemic inflammatory state after 8 weeks of intervention showed

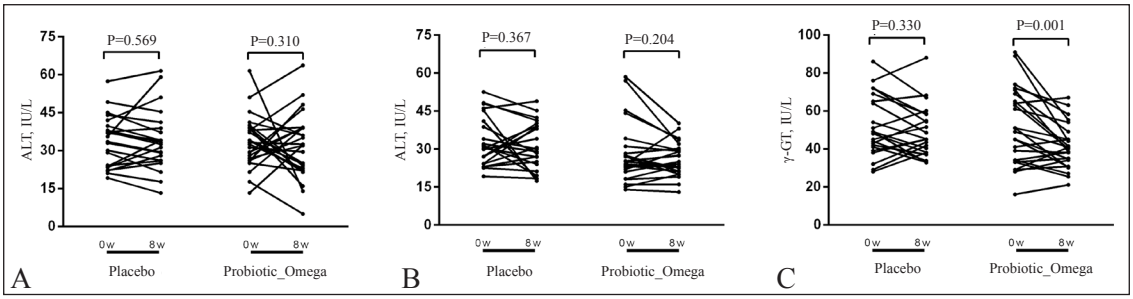

Figure 3.—Secondary outcomes analysis with accent on transaminases changes. A-C) Intra group analysis of changes at baseline and after interventon. Data expreesed as individual values at baseline and 8-week.

TABLE II.—*Changes in secondary outcomes parameters between baseline and week 8 (M±SD).*

Parameters	Placebo group (N.=22)	Probiotic-omega group (N.=26)	P
ALT, IU/L			
Absolute value	0.682±5.53	2.58±12.73	0.518
Percentage from baseline	1.07±19.42	3.20±30.38	0.779
AST, IU/L			
Absolute value	2.07±10.56	2.02±7.90	0.984
Percentage from baseline	1.67±30.98	1.35±22.66	0.969
γ-GT, IU/L			
Absolute value	2.40±11.31	10.14±14.1	0.044
Percentage from baseline	0.75±23.96	13.06±24.05	0.083
TC, mmol/L			
Absolute value	0.073±0.57	0.41±0.54	0.041
Percentage from baseline	0.5±9.9	6.44±8.37	0.039
TG, mmol/L			
Absolute value	0.29±0.89	0.75±0.79	0.066
Percentage from baseline	5.5±29.15	22.96±24.5	0.029
VLDL-C, mmol/L			
Absolute value	0.06±0.31	0.26±0.3	0.035
Percentage from baseline	2.27±25.9	17.64±23.05	0.037
HDL-C, mmol/L			
Absolute value	-0.04±0.25	-0.14±0.32	0.295
Percentage from baseline	-5.72±19.42	-13.82±28.74	0.297
LDL-C, mmol/L			
Absolute value	0.10±0.53	0.23±0.77	0.512
Percentage from baseline	1.01±17.65	3.44±22.71	0.690
TNF-α, pg/mL			
Absolute value	2.64±8.69	7.35±6.15	0.033
Percentage from baseline	3.55±18.73	14.35±12.20	0.020
IL-1β, pg/mL			
Absolute value	2.35±9.38	4.11±7.46	0.473
Percentage from baseline	-0.98±23.72	8.75±19.76	0.132
IL-6, pg/mL			
Absolute value	1.05±5.45	5.57±8.16	0.032
Percentage from baseline	-7.88±44.41	27.54±34.35	0.003
IL-8, pg/mL			
Absolute value	2.16±6.18	2.71±6.08	0.759
Percentage from baseline	7.02±22.53	7.26±27.09	0.974
IFN-γ, pg/mL			
Absolute value	4.29±35.51	22.33±59.59	0.251
Percentage from baseline	0.09±22.67	7.91±33.04	0.392

significant changes only in Symbiter Omega group: IL-1β — 38.12±15.83 *vs*. 34.01±13.65 (P=0.010) as compared to week 8, TNF-α — 49.66±17.94 *vs*. 42.31±15.59 (P<0.001), IL-8 — 28.06±7.33 *vs*. 25.35±7.78 (P=0.032), IL-6 — 14.75±11.3 *vs*. 9.18±5.98 (P=0.002), and INF-γ — 178.45±68.83 *vs*. 153.32±63.51 (P=0.016) (Figure 4A-D). However, these changes remained significant in inter-group analyses of mean changes expressed in absolute value or in percentage from baseline to end of treatment only for TNF-α and IL-6 (Table II).

Supplementation of probiotics with omega-3 FA led to a significant reduction of TG on 0.75±0.79 (P<0.001), TC - 0.41±0.54 (P=0.001), and increasing of HDL-C on 0.14±0.32 (P=0.039) respectively after 8 weeks of intervention (Figure 5A, B, D). The LDL-C level also decreased in Symbiter-Omega group but changes as compared to baseline was insignificant (Figure 5C). Mild hypolipidemic effect of probiotic-omega combination was confirmed in ANCOVA analysis. We observed significant differences for TG, TC and VLDL-C, when compare changes from baseline in placebo and probiotic group, expressed in both absolute and percent values (Table II).

Non-significant changes in serum level of transaminases, lipid metabolism parameters and

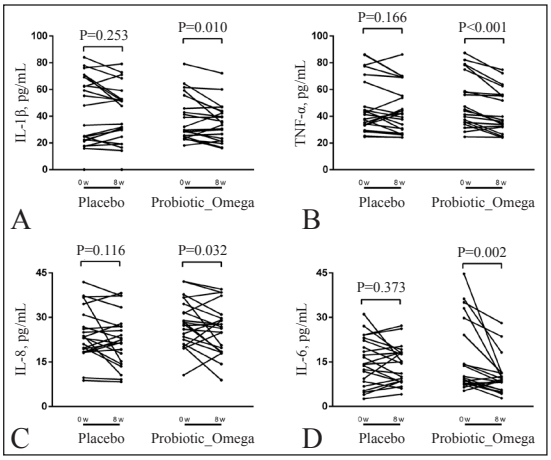

Figure 4.—Secondary outcomes analysis with accent on cytokines changes. A-D) Intra group analysis of changes at baseline and after interventon. Data expreesed as individual values at baseline and 8-week.

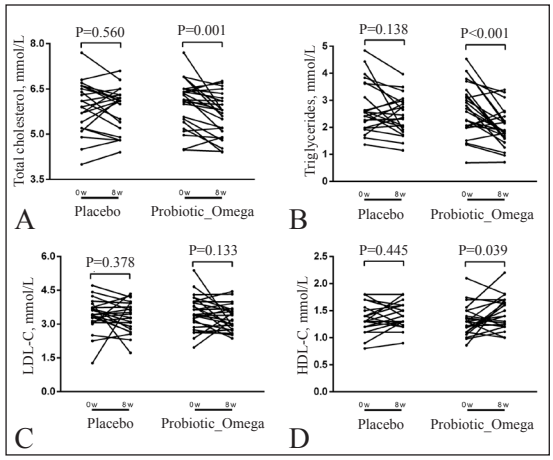

Figure 5.—Secondary outcomes analysis with accent on lipid parameters. A-D) Intra group analysis of changes at baseline and after interventon. Data expreesed as individual values at baseline and 8-week.

markers of chronic systemic inflammatory state were detected in the placebo group after intervention (Table II).

Discussion

In this RCT, we showed that coadministration of a live multi-strain probiotic mixture with omega-3 fatty acids once daily for 8 weeks to patients with NAFLD can reduce liver fat as measured by FLI, improves serum lipid levels, metabolic profile, and reduces chronic systemic inflammatory state. On the other hand, LS does not show significant changes in either group throughout the study. The outcomes utilized in the present study are in line with the ones used by Mofidi *et al.*[29] to evaluate the efficacy of symbiotic supplementation in lean NAFLD patients. In this RCT, after 28-week of administration, the mean reduction of hepatic steatosis and fibrosis according to transient elastography (Fibroscan®) was significantly greater in the symbiotic group than in the placebo (P<0.001).[29] Significant decreasing of LS, in contrast to our study, could be explained by long-term supplementation period (28 weeks).

Previously, we reported data from RCT with similar design and endpoints showing beneficial effects of probiotic formulation alone for NAFLD management.[30] After further investigations, in this study we report that additional supplementation of live probiotic strains with omega-3 FA leads to more pronounced reduction of FLI and chronic inflammatory markers. Moreover, combination of these nutraceuticals significantly improves serum lipid parameters.

The beneficial effect of probiotic supplementation containing different *Lactobacillus* and *Bifidobacterium* strains in NAFLD has been shown previously in several RCTs[29-33] and summarized in 2 recent meta-analyses on different NAFLD/NASH populations, including children and different RCT duration.[28, 29] In general, these studies showed that probiotic therapy significantly decreased transaminases level, serum levels of a marker of chronic systemic response (TNF-α), and improved lipid metabolism parameters specifically, TC and HDL.[29-37]

Long-chain (LC) n-3 poly-unsaturated fatty acids (n-3 PUFAs) have also been studied as a treatment for NAFLD. Preclinical studies demonstrated that dietary n-3 PUFAs prevent hepatic steatosis by down-regulating sterol regulatory element binding protein 1c (SREBP-1c) which is the key transcription factor of *de novo* lipogenesis and up-regulating peroxisome proliferator activated receptor (PPAR-α) which regulates genes involved in fatty acid oxidation.[38, 39] The excessive production of reactive oxygen species due to mitochondrial dysfunction causes lipid peroxidation, triggers inflammatory processes by activat-

ing redox–sensitive transcriptional factors, such as Nf-κB, and thereby causing necroinflammation and activation of stellate cells leading to fibrogenesis in NASH.[40] Recent animal studies showed that LC n-3 PUFAs supplementation decreased hepatic oxidative stress by improving the antioxidant status through restoring the antioxidant enzyme activities and glutathione level[41] and prevented high fat induced hepatic steatosis and inflammation by inhibiting NF-κB and thereby decreasing proinflammatory cytokines TNF-α and IL-1β receptors synthesis.[42, 43]

Recent RCTs indicate that in patients with metabolic syndrome, n-3 LC polyunsaturated FA supplementation in combination with protein meal replacement under calorie-restricted dietary conditions[44] or life-style modification[45] lead to significant reduction in WC and exert beneficial effects on metabolic parameters, including TG levels, HOMA-IR and IL-6.

Castro *et al.* analyzed 17 published studies investigating the effects of n-3 PUFAs on markers of NAFLD.[46] Eight studies used fish oil, one seal oil, two purified eicosapentaenoic acid (EPA), two a mixture of purified EPA and docosapentaenoic acid (DHA), one a mixture of algal EPA and DHA, two algal DHA in the absence of EPA and one a mix of flaxseed oil and fish oil. Study duration ranged from 2 to 24 month, and the daily amount of n-3 PUFAs used ranged from 250 mg DHA to 6.8 g of a mixture of EPA and DHA.[46] Authors found that twelve of these reported a decrease in liver fat and/or other markers of NAFLD after supplementation with n-3 PUFAs. Five studies also involved dietary restriction and exercise along with n-3 PUFA supplementation, however n-3 PUFAs have been proved to improve NAFLD independently of weight loss. The failure of n-3 PUFAs to decrease markers of NAFLD or other markers of inflammation in five studies may be due to short duration, poor compliance, patient specific factors and the sensitivity of the methods used.[46]

Three meta-analyses of randomized control trials of n-3 PUFA treatment of NAFLD were examined.[47-49] These meta-analyses examined 3 to 9 clinical trials each, with up to 561 total patients. There was considerable overlap of these meta-analyses with the clinical trials discussed in the previous section. The n-3 PUFA dose ranged from 0.83 to 6.4 g/d and the treatment period was 2 to 18 months in duration. The overall outcome indicates that n-3 PUFA treatment improves plasma markers of hepatic damage and TG, but yielded inconsistent effects on plasma markers for cholesterol (TC, LDL-C and HDL-C),[50] which is in agreement with data reported in this study.

Finally, Rajkumar *et al.* compared the effects of probiotics VSL#3 with omega-3 FA to single-arm therapy in apparently healthy overweight adult volunteers in a placebo controlled double-blind trial. The study showed an increase in *Lactobacilli* and *Bifidobacteria* and reduction in Gram negative bacteria with VSL#3 supplementation; improvement in insulin sensitivity and reduction in hsCRP with probiotic+omega-3 was greater than probiotic alone.[51]

Limitations of the study

Limitations of our study were the use of the US technique instead of biopsy as the diagnostic criterion for NAFLD, the small sample size, and the absence of longer-term follow up.

Conclusions

The present RCT study is the first to our knowledge to confirm in human subjects the findings previously reported in animal models: coadministration of a live multi-strain probiotic mixture with omega-3 fatty acids once daily for 8 weeks to patients with NAFLD can reduce liver fat, improve serum lipids, metabolic profile, and reduce chronic systemic inflammatory state. Considering that both probiotic and omega-3 supplementation target similar molecular pathways, implicated in hepatic lipid metabolisms, our data support the use of these combination as an intervention to attenuate dyslipidemia, chronic inflammation and liver injury associated with NAFLD. Moreover, modulation of the gut microbiota with probiotics and different nutraceuticals due to possible summation of their single positive effects may represent a more powerful branch in NAFLD management, but further studies in larger cohorts are required to determine this beneficial effect.

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