

# A Multi-strain Probiotic Reduces the Fatty Liver Index, Cytokines and Aminotransferase levels in NAFLD Patients: Evidence from a Randomized Clinical Trial

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Received: 14.12.2017  
Accepted: 02.03.2018

## ABSTRACT

**Background:** Probiotics have a beneficial effect on nonalcoholic fatty liver disease (NAFLD) in animal models. Randomized placebo-controlled trials (RCTs) in NAFLD are still lacking in humans despite a large number of data from animal research.

**Aim:** We performed a double-blind single center RCT of live multi-strain probiotic vs. placebo in type 2 diabetes patients with NAFLD.

**Methods:** A total of 58 patients met the criteria for inclusion. They were randomly assigned to receive the multi-probiotic “Symbiter” (concentrated biomass of 14 probiotic bacteria genera *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Propionibacterium*) or placebo for 8-weeks administered as a sachet formulation in double-blind treatment. The primary main outcomes were the changes in fatty liver index (FLI) and liver stiffness (LS) measured by Shear Wave Elastography (SWE). Secondary outcomes were the changes in aminotransferase activity, serum lipids and cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and IFN- $\gamma$ ) levels. Analysis of covariance was used to assess the difference between groups.

**Results:** In the probiotic group, FLI significantly decreased from 84.33 $\pm$ 2.23 to 78.73 $\pm$ 2.58 ( $p$ <0.001) but it did not change in the placebo group (82.57 $\pm$ 2.45 to 81.6  $\pm$ 2.36;  $p$ =0.367). In both groups a slight but not significant reduction of LS measured by SWE was detected. Analysis of the secondary outcomes showed that probiotics reduced the level of serum AST and GGT. Among the markers of chronic systemic inflammatory state, only TNF- $\alpha$  and IL-6 levels changed significantly after the treatment with the probiotic.

**Conclusion:** The probiotic “Symbiter” reduces liver fat, aminotransferase activity, and the TNF- $\alpha$  and IL-6 levels in NAFLD patients. Modulation of the gut microbiota might represent a new therapy for NAFLD, which should be tested in larger studies.

**Key words:** probiotics – NAFLD – cytokine – aminotransferase – fatty liver index – liver stiffness.

**Abbreviations:** ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; CFU: colony-forming unit; FLI: fatty liver index; GGT: gamma glutamyl transferase; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; MSG: mono-sodium glutamate; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; RCT: randomized placebo-controlled trials; SCFAs: short-chain fatty acids; SU: sulfonyleurea; SWE: Shear Wave Elastography; T2D: type 2 diabetes; TC: total cholesterol; TG: triglyceride; TNF- $\alpha$ : tumor necrosis factor; US: ultrasonography; WC: waist circumference.

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by the accumulation of lipids within the hepatocytes exceeding 5% of the liver weight in the absence of excessive alcohol intake and secondary causes of liver diseases [1]. NAFLD ranges from simple steatosis

to non-alcoholic steatohepatitis (NASH) that can have different degrees of fibrosis and progress to liver cirrhosis and hepatocellular carcinoma [2]. The high prevalence of obesity and type 2 diabetes (T2D) and the improved management of chronic viral hepatitis has resulted in NAFLD becoming a leading cause of chronic liver disease [3] and a major health concern responsible for hepatic and extrahepatic morbidity/mortality [4, 5].

Currently, there is no medication or surgical procedures approved for the treatment of NAFLD. However, despite the abundance of clinical trials, several pharmacological treatments

for NAFLD/NASH have been proposed, such as diabetes medications [6-9], lipid-lowering drugs [10], antioxidants [8, 9], and anti-tumor necrosis factor (TNF)- $\alpha$  agents [11].

Recently, a new treatment strategy using probiotics was proposed [12]. Data from animal studies revealed that altered microbiota may be an environmental factor that contributes to the pathogenesis of NAFLD/NASH by several mechanisms: increased production of ethanol and monosaccharide absorption, decreased consumption of vitamins and biologically active compounds, impaired production of short-chain fatty acids (SCFAs), increased inflammation, gut permeability and endotoxemia, altered lipopolysaccharide/endocannabinoid system regulatory loops and bile acids metabolism [13, 14]. The supplementation with different *Lactobacillus* and *Bifidobacterium* probiotic strains [15, 16], prebiotics [17], synbiotics (mixture of probiotics and prebiotics) [18] and their combination with nutraceuticals [19, 20] has been demonstrated to provide health benefits by decreasing hepatic tissue inflammation, reducing the hepatic triglyceride content, total body and visceral adipose tissue weight, and by improving the insulin sensitivity in different experimental NAFLD animal models.

However, despite a large body of animal evidence supporting the beneficial effect of probiotics on NAFLD, randomized placebo-controlled trials (RCTs) in NAFLD are still lacking in humans. Therefore, our aim was to perform a single center RCT of alive multistrain probiotic vs. placebo in T2D patients with NAFLD detected on ultrasonography (US). The probiotic selection was based on our previous comparative experimental analysis of different probiotic strains in NAFLD prevention [21]. In our animal study, we assessed the beneficial effects of lyophilized mono-probiotic (*B.animalis* VKL, *B.animalis* VKB, *L.casei* IMVB-7280), the combination of these three strains and multiprobiotic „Symbiter” containing biomass of 14 alive probiotic strains (*Lactobacillus* + *Lactococcus* ( $6 \times 10^{10}$  CFU/g), *Bifidobacterium* ( $1 \times 10^{10}$ /g), *Propionibacterium* ( $3 \times 10^{10}$ /g), *Acetobacter* ( $1 \times 10^6$ /g)). We have shown that short-term courses of multiprobiotic cocktails lead to significant reduction of hepatic steatosis, total lipids and triglycerides (TG) content in the liver and prevent the development of NAFLD as compared to MSG-obesity littermates. More pronounced changes were observed after the administration of the probiotic mixture preferably containing live strains as compared to lyophilized cocktails. Our data also suggested failure of NAFLD prevention with monoprobiotic strains [21].

## MATERIAL AND METHOD

The study protocol was approved by the local Ethics Committees of Kyiv City Clinical Endocrinology Center and was conducted according to the guidelines of the 1975 Declaration of Helsinki. The study was registered as Clinical.Trial.gov: NCT03434860. Prior to the study, the purpose and methodology of the study were fully explained to the participants and all the patients gave written informed consent before any study procedures were initiated.

### Study design

In this single-center double-blind, placebo controlled, parallel group study, 58 T2D patients from the Kyiv City

Clinical Endocrinology Center were selected. They were randomly assigned to receive the multiprobiotic “Symbiter” or placebo for 8 weeks, administered as a sachet formulation in double-blind treatment. The study had a 1:1 allocation ratio. Randomization was done by the study statistician with blocks of four using a computer-generated list ([www.randomization.com](http://www.randomization.com)). The groups were homogeneous according to age, sex and diagnosis criteria. Sachets containing either the probiotic or a placebo were identical in terms of nutritional value, appearance, texture, weight and smell and were only differentiated by a code (“A” or “B”) placed on them. The study pharmacist gave the sachets to the participants according to their group assignment and was responsible for the delivery of the blinded supplements. The assignment of groups was blind to the participants, research staff and outcome assessors. Moreover, to maintain a blind parallel study the statistician was not aware of the allocation of participants to intervention. Code breaking was performed after the analysis was completed and the database was locked.

The multiprobiotic „Symbiter” was supplied by the Scientific and Production Company „O.D. Prolisok” (Kyiv, Ukraine). It contained 14 alive probiotic strains of *Lactobacillus* + *Lactococcus* ( $6 \times 10^{10}$  CFU/g), *Bifidobacterium* ( $1 \times 10^{10}$ /g), *Propionibacterium* ( $3 \times 10^{10}$ /g), *Acetobacter* ( $1 \times 10^6$ /g) genera. Over 8 weeks of the interventional period, the patient received 1 sachet (10 grams) of probiotic or placebo per day. All sachets were identical with similar organoleptic characteristics (e.g., taste and appearance). Administration instructions were provided with each pack. All patients were instructed to take the trial medication as prescribed.

The pre-randomization period was designed to minimize the effects of dietary changes on the metabolic markers. For this purpose, two weeks before the study was initiated, after signing the inform consent, the patients were instructed in one-to-one sessions with a dietitian to follow a therapeutic lifestyle-change diet as classified by the NCEP. In addition, participants were instructed to continue with a stable anti-hyperglycemic treatment and received standardized mild physical training for 1 hour per day.

Throughout the study, weekly phone follow-up visits were provided for the 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 (aged 18–65, BMI  $\geq 25$  kg/m<sup>2</sup>) diagnosed with NAFLD according to the recommendations of the American Gastroenterology Association (AGA) and the American Association for the Study of Liver Disease (AASLD) on the basis of clinical examination, laboratory values of lipid and carbohydrate metabolism, liver enzyme activities (alanine aminotransferase - ALT, aspartate aminotransferase - AST), ALT/AST ratio, and ultrasound (US) examination [5, 22] were included in the study. The diagnosis of fatty liver was based on the results of abdominal ultrasonography, which was performed by trained technicians with Ultima PA (Radmir Co., Kharkiv, Ukraine). Of 4 known US criteria for NAFLD (hepato-renal echo contrast, liver brightness, deep

attenuation, and vascular blurring), the diagnosis of NAFLD in our patients was formulated if hepato-renal contrast and liver brightness were detected [5]. Individuals with T2D treated with diet and exercise alone or metformin, SUs and insulin were included, as well as individuals with AST and ALT  $\leq 3$ x upper limit of normal. Eligible participants received the antidiabetic drug at a stable dose at least 4 weeks prior to the start of the study.

#### Exclusion criteria

The main exclusion criteria included alcohol abuse ( $>20$  g/day, i.e. 2 standard drinks in women or  $>30$  g/d i.e. 3 drinks in men over a two-year period), chronic viral hepatitis (HBV, HCV, HDV), drug-induced liver disease, Wilson's disease, hereditary deficiency of antitrypsin-1 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 the fatty liver index (FLI) and liver stiffness (LS) measured by Shear Wave Elastography (SWE).

Liver stiffness was measured by SWE using a multifrequency convex transducer (2-5 MHz) and Ultima PA ultrasound equipment (Radmir, Ukraine). First, the liver was examined in B-mode: the ratio of the edge to the costal arch, the availability of acoustic windows, the antero-posterior size on inspire for liver lobes, the contour, 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 [23]. Shear Wave Elastography was carried out by the standard algorithm. We performed 10 valid measurements of LS in every patient, and a median value was calculated, the result being expressed as kPa.

The FLI, a validated prediction score for hepatic steatosis severity designed by Bedogni et al. [24] was calculated using their formula based on laboratory and anthropometric measures, including triglycerides and gamma glutamyl transferase (GGT) levels, body mass index (BMI), and waist circumference (WC).

Secondary outcomes were the changes in aminotransferase activity, serum lipids and cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and IFN- $\gamma$ ) levels. All values were determined at the hospital clinical laboratory following a 12-h fasting period.

Anthropometric data including weight and height were measured to an accuracy of 0.1 kg and 0.5 cm, respectively. Body mass index (BMI) was calculated (weight/(height<sup>2</sup>). Waist (narrowest diameter between xiphoid process and iliac crest) circumferences (WC) were measured as well.

Serum activity of alanine (ALT) and aspartate aminotransferase (AST) was 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 [25].

The contents in serum interleukins (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and IFN- $\gamma$ ) were measured by ELISA using specific mono- and polyclonal antibodies (Sigma). The studied molecules were immobilized in 96-well plates with sorption surface.

#### Statistical analysis

The SPSS statistical package, version 20.0 (SPSS, Inc., Chicago, Illinois) 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  $\pm$  standard deviation (M $\pm$ SD) or %. Data distribution of continuous variables was analyzed using the Kolmogorov-Smirnov normality test. The baseline characteristics of participants in the two groups were compared using independent sample *t*-tests and chi-squared ( $\chi^2$ ) or Fisher exact 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 two groups after intervention, adjusting for baseline measurements and confounders (BMI and sex).

## RESULTS

A total of 58 patients were randomly divided into two groups receiving either probiotic (n=30) or placebo (n=28), respectively. All subjects completed the study and received more than 90% of prescribed sachets. Both probiotic and placebo were well tolerated and during the study period the participants reported only several minor adverse events. In the probiotic group one patient complained of short-term diarrhea and another one of mild headaches. In the placebo group the main adverse events were also gastrointestinal symptoms. Two patients reported mild abdominal pain and one complained of nausea. However, the prevalence of adverse events was comparable between the groups (placebo=10.7 % vs probiotics=6.7 %, Fisher exact test, p=0.665) and did not result in the withdrawal of patients from the study.

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

The FLI of subjects at baseline and after the 8-week intervention are presented in Fig. 1. In the probiotic group our primary endpoint, FLI, significantly decreased from  $84.33 \pm 2.23$  to  $78.73 \pm 2.58$  (p<0.001), but without changes in the placebo group ( $82.57 \pm 2.45$  to  $81.6 \pm 2.36$ ; p=0.367) (Fig.

**Table I.** Anthropometric, clinical and laboratory parameters in the study patients (M±SD)

Parameters	Placebo group (n=28)	Probiotic group (n=30)	P
Age, years	57.29±10.45	53.4±9.55	0.145
Duration of T2D, years	5.25±2.83	7.03±5.85	0.150
Metformin, % (n)	71.4 (20)	70.0 (21)	0.905
Metformin daily dosage, mg	1837.5±110.67	1892.86±103.18	0.716
Sulfonilureas, % (n)	53.6 (15)	43.3 (13)	0.436
Insulinotherapy, % (n)	25.0 (7)	36.7 (11)	0.337
Insulin daily dosage, IU	38.14±11.94	33.91±12.72	0.323
BMI, kg/m <sup>2</sup>	34.26±6.17	34.82±6.84	0.746
Weight, kg	94.5±15.29	98.1±13.78	0.350
Waist circumference, cm	95.21±6.49	97.53±5.81	0.157
FLI	82.57±12.97	84.33±12.22	0.596
LS, kPa	7.28±1.19	7.16±1.09	0.686
ALT, IU/L	39.48±17.90	38.18±15.75	0.771
AST, IU/L	42.7±19.23	38.77±15.42	0.393
γ-GT, IU/L	46.44±15.3	51.73±20.73	0.277
TC, mmol/l	6.07±0.85	6.28±0.89	0.367
TG, mmol/l	2.68±0.9	2,57±1.03	0,679
VLDL-C, mmol/l	1.17±0.37	1.16±0.49	0.954
HDL-C, mmol/l	1.39±0.25	1.34±0.23	0.415
LDL-C, mmol/l	3.45±0.74	3.78±0.79	0.111
TNF-α, pg/ml	49.37±19.09	51.18±19.48	0.723
IL-1β, pg/ml	43.56±22.62	41.05±19.16	0.650
IL-6, pg/ml	13.7±9.21	16.74±14.19	0.340
IL-8, pg/ml	25.83±10.4	29.13±8.88	0.199
IFN-γ, pg/ml	165.52±76.9	185.24±71.97	0.318

FLI: fatty liver index; LS: liver stiffness; TC: total cholesterol; TG: triglyceride; ALT: alanine aminotransferase; AST: aspartate aminotransferase, GGT: gamma glutamyl transferase; T2D: type 2 diabetes

1A, B). We also observed significant differences between the mean changes of FLI expressed in absolute value (Fig. 1C) or percentages (Fig. 1D) from baseline to the end of treatment in ANCOVA analysis.

In both interventional groups a slight insignificant reduction of LS measured by SWE were detected (2A-D). Therefore, LS from baseline to the end of treatment decreased in the probiotic group (7.16±0.2 to 6.76 ±0.22; p=0.052) more pronounced when compared to the placebo group (7.28±0.22 to 7.14±0.26; p=0.396) (Fig. 2A, B).

Analysis of secondary outcomes (Table II) evidenced that probiotic intake significantly reduced the level of serum AST - 12.6 % (p<0.001) and GGT - 12.0 % (p=0.001), but not ALT (p=0.562) (Fig. 3A-F). Moreover, these changes remained significant at inter-group analysis of mean changes expressed in absolute value or in percentage from baseline to the end of treatment. In the placebo group, the serum level of aminotransferases, lipid metabolism parameters and markers of chronic systemic inflammatory state, after intervention changed insignificantly (Table II).

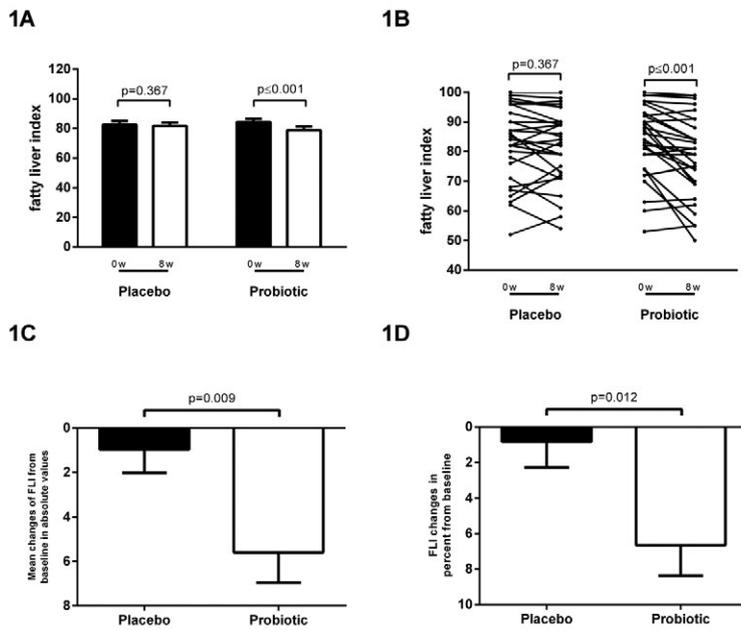
**Table II.** Changes in the secondary outcomes parameters between baseline and week 8 (M±SD)

Parameters	Placebo group (n=28)	Probiotic group (n=30)	p
ALT, IU/L			
Absolute value	0.8±6.76	1.0±9.39	0.508
% from baseline	-0.73±19.79	1.14±23.33	0.503
AST, IU/L			
Absolute value	1.4±12.78	5.79±7.03	0.014
% from baseline	1.25±30.39	12.58±15.19	0.041
γ-GT, IU/L			
Absolute value	0.18±10.86	10.6±15.43	0.003
% from baseline	-2.18±25.76	11.98±28.23	0.014
TC, mmol/l			
Absolute value	0.18±0.47	0.38±0.58	0.236
% from baseline	2.57±7.97	5.53±8.77	0.253
TG, mmol/l			
Absolute value	0.24±0.84	0,58±0,89	0.133
% from baseline	4.26±27.77	14.0±33.56	0.074
VLDL-C, mmol/l			
Absolute value	0.08±0.32	0.12±0.30	0.597
% from baseline	3.26±26.19	3.94±31.17	0.657
HDL-C, mmol/l			
Absolute value	-0.02±0.21	-0.07±0.27	0.504
% from baseline	-3.26±16.17	-7.65±23.48	0.586
LDL-C, mmol/l			
Absolute value	0.05±0.47	0.35±0.61	0.088
% from baseline	-0.52±16.27	7.34±16.48	0.120
TNF-α, pg/ml			
Absolute value	1.17±11.22	7.53±7.29	0.040
% from baseline	1.26±22.10	13.98±13.12	0.027
IL-1β, pg/ml			
Absolute value	0.52±7.66	2.47±9.24	0.432
% from baseline	-2.54±20.45	1.61±21.68	0.335
IL-6, pg/ml			
Absolute value	0.39±5.83	4.63±7.84	0.003
% from baseline	-15.4±43.20	15.08±41.58	0.004
IL-8, pg/ml			
Absolute value	2.41±6.38	1.79±6.82	0.797
% from baseline	6.37±20.96	1.82±25.64	0.767
IFN-γ, pg/ml			
Absolute value	4.89±32.45	11.37±61.72	0.253
% from baseline	0.12±21.88	-0.92±37.69	0.304

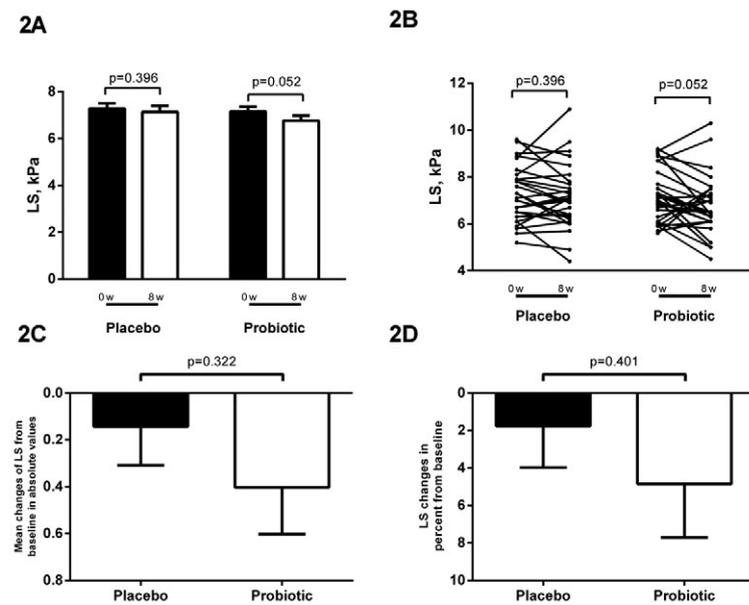
See Table I for abbreviations.

Similarly, the probiotic lead to significant decreasing of TG on 0.58±0.16 (p=0.001), TC - 0.38±0.10 (p=0.001), LDL-C - 0.35±0.11 (p=0.004) and VLDL on 0.12±0.05 (p=0.033) levels, respectively, after 8 weeks of intervention (Fig. 4A-H). Therefore, when we compared the mean changes from baseline to the end of treatment in the placebo and probiotic group, we observed significant differences only for the TG level; other lipid parameters did not change significantly (Table II).

Among the markers of chronic systemic inflammatory state, only TNF-α (7.53±1.33 pg/ml, p<0.001) and IL-6 levels (4.63±1.43 pg/ml, p=0.003) changed significantly after 8 weeks of treatment with probiotics. The ANCOVA analysis also evidenced statistically significant differences between the two groups for mean changes in terms of TNF-α and IL-6 levels. Other cytokines levels did not change significantly in both interventional groups (Fig. 5A-H).



**Fig. 1.** Primary outcomes analysis with the accent on FLI changes. A, B – intra-group analysis of changes at baseline and after intervention. 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±SEM. ANCOVA was used to identify any differences between the two groups after intervention.



**Fig. 2.** Primary outcomes analysis with the accent on LS changes. A, B – intra-group analysis of changes at baseline and after intervention. 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±SEM. ANCOVA was used to identify any differences between the two groups after intervention.

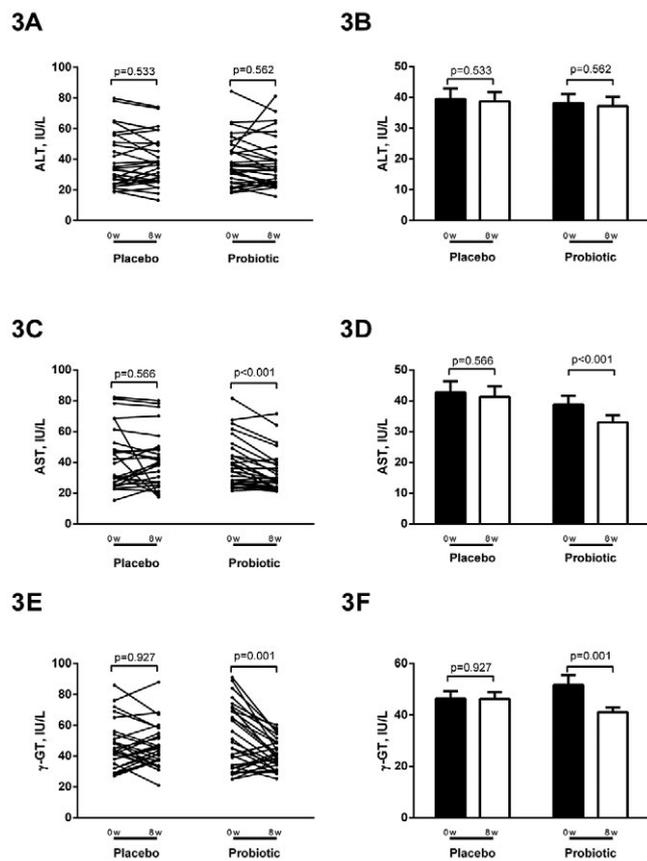
## DISCUSSION

The present randomized placebo-controlled single-center study used, for the first time to our knowledge, the FLI and LS measured by SWE as the main primary outcomes for assessment of the efficacy of alive probiotic when administered once daily for 8 weeks in patients with NAFLD. The data generated by the study indicates that supplementation with the probiotic lead to significant decreasing of FLI, as compared to the placebo group.

The reduction of hepatic fat content, as measured by proton-magnetic resonance spectroscopy, was also observed in biopsy-proven NAFLD patients after 6-months of treatment with Lepicol probiotic formula (*Lactobacillus plantarum*, *Lactobacillus deslbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum*)

[26]. According to the study of Wong et al. [26], probiotic supplementation leads to significant decrease of intrahepatic triglyceride content (IHTG) after intervention, but remains static in the usual care group. Moreover, 60% of probiotic-treated patients had IHTG reduced by more than 30% from baseline [26].

In respect to another primary outcome, a slight not significant reduction of LS was mentioned in both interventional groups, but it was more pronounced after the probiotic administration. Mofidi et al. [27] used, similar to the present study, transient elastography (FibroScan®) with hepatic steatosis (CAP score) measurements (quantitative, non-invasive alternative to biopsy), to evaluate the efficacy of synbiotic supplementation in NAFLD patients. In the randomized, double-blind, placebo-controlled, clinical trial hepatic steatosis and fibrosis reduction was observed in both groups; however, the mean reduction

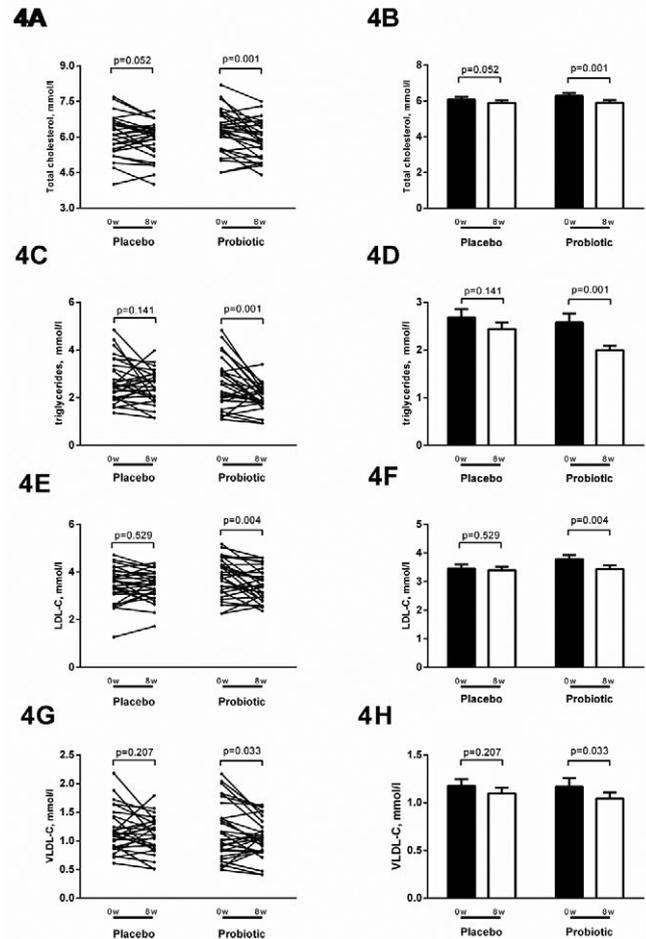


**Fig. 3.** Secondary outcomes analysis with the accent on aminotransferases changes (A, B -ALT; C, D - AST; E, E - GGT). A-F – intra-group analysis of changes at baseline and after intervention. Data expressed as individual values (A, C, E) or mean±SEM (B, D, F) at baseline and after 8 weeks of treatment.

was significantly greater in the synbiotic group rather than in the placebo group ( $p<0.001$ ) [27]. The significant decrease of LS, in contrast to our study, could be explained by a long-term supplementation period (28 weeks).

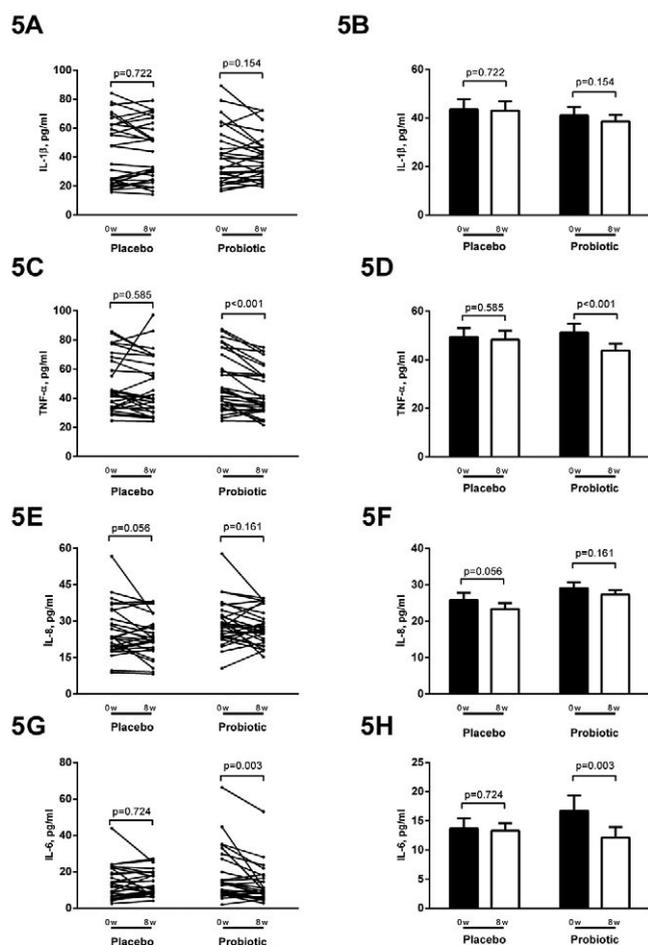
The efficacy of probiotic supplementation containing different *Lactobacillus* and *Bifidobacterium* strains in NAFLD has been shown previously. Two recent meta-analyses which included 4 RCT involving 134 NAFLD/NASH patients [28] and 9 RCT with a total of 535 cases of NAFLD [29], respectively, showed that the probiotic therapy significantly decreased ALT, AST, TC, HDL and TNF- $\alpha$ , as compared with the placebo group, with variations in different patient populations. Furthermore, a meta-analysis reported by Gao et al. [29], additionally to the meta-analysis done by Ma et al. [28], included NAFLD children studies and TG analysis. Statistical differences in LDL, ALT, AST and BMI were detected between the two children groups ( $p\leq 0.05$ ) [29]. The value of TG significantly decreased after probiotic administration only in Italian and Spanish NAFLD patients [29]. In contrast, in our study, a significant decrease of TG, TC, LDL-C and VLDL was detected after 8 weeks of intervention with probiotics, but not placebo. These changes, however, remained significant only for the TG value in the inter-group analyses when we compared mean changes from baseline using ANCOVA.

In our study, we included AST, ALT and GGT levels in the secondary outcomes assessment analysis. We observed that the probiotics significantly reduced the activity of



**Fig. 4.** Secondary outcomes analysis with the accent on lipid parameters (A, B – total cholesterol; C, D – triglycerides; E, F – LDL-cholesterol; G, H – VLDL-cholesterol). A-H – intra-group analysis of changes at baseline and after intervention. Data expressed as individual values (A, C, E, G) or mean±SEM (B, D, F, H) at baseline and after 8 weeks of treatment.

AST and GGT, but not of ALT ( $p=0.562$ ). Our data is in accordance with the studies reported by Malaguarnera et al. [30] and Mofidi et al. [27], in which significant reductions only in the serum AST level were observed. Interestingly, supplementation with synbiotics was reported in both cases. In Malaguarnera et al. [30] study, subjects with NAFLD received a capsule containing *Bifidobacterium longum* with fructo-oligosaccharides. Mofidi et al. [27] used Protexin synbiotic capsule containing 200 million bacteria of seven strains (*Lactobacillus*, *Streptococcus thermophilus*, *Bifidobacterium*), prebiotic (125 mg fructo-oligosaccharide) and some mineral and vegetable (hydroxypropylmethyl cellulose). Loguercio et al. (2002), in a pilot study, reported that synbiotic intervention with several species of *Lactobacillus* (*acidophilus*, *bifidus*, *rhamnosus*, *plantarum*, *salivarius*, *bulgaricus*, *lactis*, *casei*, and *breve*) mixed with prebiotic fructo-oligosaccharide and some vitamins decreased serum concentrations only of ALT in patients with NAFLD [31]. In contrast to our study, only a few clinical trials on the use of probiotics containing different strains of *Lactobacillus* and *Bifidobacterium* in NAFLD in adults (Nabavi et al. 2014; Aller et al. 2011) [32, 33] and one in children (Famouri et al. 2017) showed significant reductions in both serum ALT and AST [34].



**Fig. 5.** Secondary outcomes analysis with accent on cytokines changes (A, B - IL-1 $\beta$ ; C, D - TNF- $\alpha$ ; E, F - IL-8; G, H - IL-6). A-H - intra-group analysis of changes at baseline and after intervention. Data expressed as individual values (A, C, E, G) or mean $\pm$ SEM (B, D, F, H) at baseline and after 8 weeks of treatment.

Among the markers of chronic systemic inflammatory state only TNF- $\alpha$  and IL-6 levels changed significantly in both inter- and between group analysis. Previous studies have shown that intestinal bacteria, by enhancing intestinal permeability [35], determine a direct activation of inflammatory cytokines via release of lipopolysaccharide (LPS). This can result in the production of free radical species in the liver, which might contribute to the development of NAFLD and NASH [27, 28]. The endotoxins activate Kupffer cells in the liver and increase the production of TNF- $\alpha$  and IL-6, which contribute to the onset of liver fibrosis [36]. Li et al. [37] reported that probiotics improved liver histology, reduced the hepatic total fatty acid content via decreased hepatic activity of JNK, a TNF-regulated stress kinase that promotes hepatic insulin resistance. Our study also supports the fact that probiotics have a positive effect on reducing TNF- $\alpha$  and IL-6 levels in NAFLD patients.

The general 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 a longer-term follow up.

Another possible confounder which could have biased the final results interpretation is the use of metformin. In light of the

latest evidence, metformin is known to have pleiotropic effects beyond glucose reduction, including improvement of lipid profiles, GLP-1, bile acids and gut microbiota [38]. Metformin treatment increased the relative abundance of *Akkermansia muciniphila* and *Clostridium cocleatum* in a mouse model of high-fat diet [39]. Moreover, in a double-blind RCT, treatment of naïve T2D mice for 4 months showed that metformin had strong effects on the gut microbiome, as compared to a placebo. Transfer of fecal samples from metformin-treated donors to germ-free mice improved glucose tolerance in the mice which received metformin-altered microbiota [40]. To avoid the impact of metformin, in our study we randomized equal proportions of patients, treated with a stable dose of drug at least 4 weeks prior to the study start. Moreover, the mean dosage of metformin was well balanced between the probiotic and the placebo group (Table I).

## CONCLUSION

Our data in humans corroborate the findings in previous pre-clinical studies. Our clinical trial demonstrated that the probiotic Symbiter can significantly reduce liver fat, aminotransferase activity, and TNF- $\alpha$  and IL-6 levels in NAFLD patients. Modulation of the gut microbiota represents a new treatment for NAFLD, and long-term supplementation of probiotics, as well as metabolomic analysis of gut microflora should be tested in larger studies.

**Conflicts of interest:** None to declare.

**Authors' contributions:** N.K. and L.A. conceived and designed the study. N.K., G.M., L.B., L.K., D.K. and O.D. wrote the manuscript. All authors enrolled patients and approved the final version of the manuscript.

**Acknowledgements:** The authors express their sincere thanks to Dr. Yankovsky Dmitro Stanislavovych for the help, advice and financial support of this work.

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