Effects of long-term synbiotic supplementation in addition to lifestyle changes in children with obesity-related non-alcoholic fatty liver disease

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ABSTRACT
Background/Aims: We aimed to analyze the efficiency of a novel treatment approach, long-term synbiotic supplementation, in addition to lifestyle changes in children with non-alcoholic fatty liver disease (NAFLD).

Materials and Methods: The study included children with NAFLD (n=28) and a healthy control group (n=30). Children with NAFLD were given 1 capsule/day of synbiotics. Anthropometric parameters; biochemical analysis, including ethanol, tumor necrosis factor-α (TNF-α), total oxidant status (TOS) and anti-oxidant status (TAS), zonulin, and fecal calprotectin; and ultrasonographic examination were performed at baseline and 4 months later.

Results: The grade of fatty liver was decreased (≥1 grade) in 19 of the 28 patients (67.8%) after synbiotic supplementation. Total cholesterol, low-density lipoprotein (LDL) levels, TNF-α, C-reactive protein (CRP), and ethanol were significantly decreased, and TAS levels were significantly increased at the end of treatment (p<0.05 for all). We found that the median decrease in CRP (-0.16 vs. -0.03 mg/dL, p=0.003) and LDL levels (-17 vs. -3 mg/dL, p=0.019) were higher in patients who responded to the supplementation.

Conclusion: Synbiotic supplementation in addition to lifestyle changes is effective in children with NAFLD.

Keywords: Non-alcoholic fatty liver disease, synbiotics, treatment, ethanol
ance and low-grade efficiency indicate the use of the combination with pharmacological medications (3). Various pharmacological medications have been used in addition to lifestyle modifications, but drawing a firm conclusion regarding the efficiency of these medications was challenging (6). Therefore, we aimed to analyze the efficiency of a novel treatment approach, long-term synbiotic supplementation, in addition to lifestyle changes in children with NAFLD.

MATERIALS AND METHODS
This was a longitudinal study of 4 months including patients with NAFLD (n=30) and healthy children (n=30). The diagnosis of NAFLD was made based on the physical findings (obesity) along with sonographic findings of fatty liver disease and/or elevated liver enzymes (alanine aminotransaminase [ALT] >45 U/L) (1). Patients were excluded if they had other causes of liver diseases. Patients were also excluded if they had any other known systemic diseases, consumed alcohol, or used any drugs for the treatment of NAFLD, and previously and currently used pre- and/or probiotics and antibiotics for any reason within 6 months.

Children with NAFLD were given 1 capsule/day of synbiotics (Maflor plus capsules®) that contained 7×10⁹ colony-forming unit (CFU) active probiotics (Bifidobacterium lactis, Lactobacillus acidophilus, and Lactobacillus casei) and 100 mg chicory inulin for 4 months. In addition to the medical treatment, patients were prescribed a low-calorie diet (approximately 10%-20% low calorie according to their age with 50%-60% carbohydrates, 20%-30% fat: two-third saturated and one-third unsaturated, and 10%-20% protein) and a moderate exercise program (aerobic exercise 30-45 min/d at least 3 times a week). None of the patients received antibiotics during the follow-up. Compliance to diet and exercise program was checked at each visit by face-to-face questionnaire the second author of the manuscript.

Anthropometric parameters were analyzed, and biochemical analysis and ultrasonographic examination were performed at baseline and 4 months later in all (patients). Body mass index (BMI) and standard deviation scores were calculated using the Turkish reference data (7). Total body fat level was measured using a Tanita BC 418® device (Tanita, Tokyo, Japan). Turkish reference data (7). Total body fat level was measured using a Tanita BC 418® device (Tanita, Tokyo, Japan).

Venous blood samples collected after an overnight 12-hour fast were used to measure biochemical parameters, including glucose, liver enzymes, insulin, plasma lipids (total cholesterol [TC], triglycerides [TG], high-density lipoprotein [HDL], low-density lipoprotein [LDL], and very low density lipoprotein [VLDL]), liver enzymes, C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), serum ethanol, total oxidant and anti-oxidant status (TOS and TAS), and zonulin. Stool samples were collected for the fecal calprotectin analysis. The degree of insulin resistance/sensitivity was estimated using the homeostatic model assessment (HOMA-IR) equation as follows: (fasting insulin×fasting glucose)/405 (Table 1) (8).

### Table 1. Parameters used in the study

<table>
<thead>
<tr>
<th>Indications</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthropometry</td>
<td>Weight, BMI*, total body fat</td>
</tr>
<tr>
<td>Liver steatosis and inflammation</td>
<td>Ultrasound, ALT, AST</td>
</tr>
<tr>
<td>Systemic inflammation</td>
<td>CRP, TNF-α</td>
</tr>
<tr>
<td>Metabolic status</td>
<td>Ethanol, TC, TG, HDL, LDL, VLDL, glucose, HOMA-IR</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>TAS, TOS</td>
</tr>
<tr>
<td>Intestinal permeability</td>
<td>Zonulin</td>
</tr>
<tr>
<td>Intestinal inflammation (bacterial overgrowth)</td>
<td>Fecal calprotectin</td>
</tr>
</tbody>
</table>

Biochemical Measurements
A part of the collected blood was centrifuged and stored at -80°C for further analysis of the levels of TNF-α, TAS, TOS, and zonulin. Specific sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of TNF-α and zonulin (TNF-α-EASIA Kit, DIAsource, Belgium, and CusaBio, China, respectively). TOS of serum was determined using a novel automated measurement method as previously described (9). Serum TOS levels were calculated in µmol hydrogen peroxide (H₂O₂) equivalent/L. TAS of the serum was determined using a novel automated measurement method developed by Erel (10). Serum TAS levels were calculated in mmol Trolox equivalent/L. Stool samples were stored at -20°C for the further analysis of calprotectin levels. Sandwich ELISA was used for the analysis of calprotectin levels using IDK® Calprotectin ELISA kits.

Liver Ultrasound
Liver ultrasound was performed by the same experienced radiologist using a Toshiba Apio A500 TUS scanner equipped with 6C1 convex probe. Liver steatosis was graded as normal (grade 0), mild (grade 1), moderate (grade 2), and severe (grade 3) according to sonographic findings as defined elsewhere (11). The response to the treatment was defined as decrease in liver steatosis ≥1 grade at end of 4 months compared to baseline.

Statistical Analysis
Statistical analysis was performed using Statistical Package for Social Sciences version 23 (IBM Corp.; Armonk, NY, USA). Differences between groups were calculated using an independent samples t-test (Student’s t-test) for the normally distributed data and the Mann-Whitney U test for data that was not normally distributed. Chi-square test was used for the comparisons of qualitative data. Correlations between variables were calculated using linear regression. Values of p<0.05 were considered significant.
Informed consent for participating in the study was obtained from parents of all cases and approved by the ethics committee (2014/97). The study was funded by the Scientific Research Projects Unit (TTU-2015-5321).

RESULTS

Thirty patients were planned to be included the study but two patients were lost to follow-up during the study period, and final analysis were performed in 28 patients. Table 2 shows the baseline anthropometric parameters and biochemical measurements of the children with NAFLD and the healthy control group.

As expected, BMI, BMI Z-score, total body fat, serum ALT and aspartate aminotransaminase (AST), TC, TG, LDL, VLDL, and HOMA-IR levels were higher in children with NAFLD compared to healthy controls at baseline (p<0.05 for all). Additionally, serum ethanol, CRP, TNF-α, zonulin levels, and fecal calprotectin levels were higher in patients with NAFLD compared to healthy controls (p<0.05 for all). In the complete study group (n=58), ALT levels positively correlated to serum ethanol levels (p=0.04, r=0.377). In addition, ethanol levels positively correlated to TNF-α levels (p=0.003, r=0.368), and fecal calprotectin levels positively correlated to zonulin levels (p=0.0001, r=0.683; Figure 1).
Table 3. Effects of synbiotic supplementation on TNF-α, CRP, ethanol, zonulin, TAS, TOS, and fecal calprotectin levels

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline (mean±SD)</th>
<th>At the end of the treatment (mean±SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (mg/dL)</td>
<td>4.93±3.95</td>
<td>0.92±1.43</td>
<td>0.001</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>0.51±0.44</td>
<td>0.39±0.38</td>
<td>0.003</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>9.92±7.48</td>
<td>8.46±7.44</td>
<td>0.01</td>
</tr>
<tr>
<td>TAS (mmol troloxEq/L)</td>
<td>1.43±0.35</td>
<td>1.84±0.43</td>
<td>0.02</td>
</tr>
<tr>
<td>TOS (mmol troloxEq/L)</td>
<td>15.1±2.78</td>
<td>15.27±28.18</td>
<td>0.9</td>
</tr>
<tr>
<td>Zonulin (ng/mL)</td>
<td>3.3±2.2</td>
<td>3.4±1.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Fecal calprotectin (ng/mL)</td>
<td>11.8±11.4</td>
<td>10.8±7.7</td>
<td>0.714</td>
</tr>
</tbody>
</table>

CRP: C-reactive protein; TAS: total antioxidant status; TNF-α: tumor necrosis factor-α; TOS: total oxidant status.

Table 4. Changes in some parameters from baseline to the end of the treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Responsive; median (min, max)</th>
<th>Non-responsive; median (min, max)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>-2.3 (-4.3, 0.3)</td>
<td>-0.9 (-2.9, 1.9)</td>
<td>0.049</td>
</tr>
<tr>
<td>BMI Z-score</td>
<td>-0.18 (-0.53, 0.01)</td>
<td>-0.02 (-0.15, 0.28)</td>
<td>0.022</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>-4 (-12.3, 0.9)</td>
<td>-3 (-8.2, 2.6)</td>
<td>0.243</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>-7 (-137.8)</td>
<td>-1 (-118, 240)</td>
<td>0.468</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>-5 (-56.6)</td>
<td>-4 (-60, 130)</td>
<td>0.699</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>-18 (-42.8)</td>
<td>-2 (-30, 43)</td>
<td>0.09</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>-1 (-98.150)</td>
<td>-37 (-244, 99)</td>
<td>0.290</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>-17 (-52, 24)</td>
<td>-3 (-24, 45)</td>
<td>0.290</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>-1 (-19, 22)</td>
<td>-4 (-49, 28)</td>
<td>0.629</td>
</tr>
<tr>
<td>CRP* (mg/dL)</td>
<td>0.51±0.44</td>
<td>0.39±0.38</td>
<td>0.003</td>
</tr>
<tr>
<td>TOS (mmol troloxEq/L)</td>
<td>15.1±2.78</td>
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CRP: C-reactive protein; TAS: total antioxidant status; TNF-α: tumor necrosis factor-α; TOS: total oxidant status.

Effects of Synbiotics on Fatty Liver and Liver Enzymes

The grade of fatty liver was decreased (≥1 grade) in 19 of the 28 patients (67.8%, 95% confidence interval [CI]: 50.49-85.11) 4 months after the synbiotic supplementation. At the end of the 4 months, 3 patients (10.7%) had normal echo, 11 had (39.3%) grade 1, 12 had (42.9%) grade 2, and 2 had (7.1%) grade 3 steatosis (p<0.05 when compared to baseline parameters of 0%, 17.9%, 57.1%, and 25%, respectively; Figure 2). Additionally, the likelihood of "normal+grade 1 fatty liver" was increased from 17.9% (n=5) to 50% (n=14) at the end of the treatment (p=0.01, odds ratio [OR]: 4.6, 95% CI: 1.36-15.5).

After synbiotic supplementation, serum ALT and AST levels were significantly decreased from 35.58±21.73 U/L to 35.58±21.73 U/L (p=0.001) and from 45±22.9 U/L to 41.46±37.95 (p=0.02), respectively. Normalization of ALT (<45 U/L) was observed in 9 (60%) of 15 patients whose ALT levels were previously high.

Effects of Synbiotics on Anthropometric Parameters, Lipid Levels, and HOMA-IR

BMI, BMI Z-score, and total body fat were significantly decreased after synbiotic supplementation (30.27±5.97 vs. 28.73±5.47 kg/m², p=0.001; 20.9±3.73 vs. 19.7±4.82, p=0.001; and 34.1±6.33 vs. 30.1±3.79, p=0.001, respectively). TC (173.75±26.79 mg/dL vs. 161.64±26.3 mg/dL, p=0.005) and LDL levels (101.46±24.55 mg/dL vs. 89.6±20.4 mg/dL, p=0.005) were decreased after the supplementation but no improvement was observed in TG, HDL, VLDL, and HOMA-IR levels.

Effects of Synbiotics on Other Parameters

Effects of synbiotic supplementation on TNF-α, CRP, ethanol, zonulin, TAS, TOS, and fecal calprotectin are shown in Table 3. Levels of TNF-α (9.92±7.48 pg/mL vs. 8.46±7.44 pg/mL, p=0.01), CRP (0.51±0.44 mg/dL vs. 0.39±0.38 mg/dL, p=0.003), and ethanol (4.93±3.95 mg/dL vs. 0.92±1.43 mg/dL, p=0.001) were decreased, and TAS levels (1.43±0.35 mmol troloxEq/L vs. 1.84±0.43 mmol troloxEq/L, p=0.02) were increased at the end of the treatment, but no significant difference was found in the remaining parameters.

Baseline characteristics of the patients who responded to the supplementation (n=19) were compared with unresponsive patients (n=9) and found that baseline ALT levels were low (median, range: 50 U/L, 14-167 U/L vs. 41.46±37.95 (p=0.02), respectively. Normalization of ALT (<45 U/L) was observed in 9 (60%) of 15 patients whose ALT levels were previously high.

A post-hoc power analysis of our findings revealed that the power of our study was 86.3% (α=0.05, expected beneficial effect of probiotics=40%, sample size=28).
DISCUSSION

In this study, we found that (i) apart from anthropometric parameters, liver enzymes, and lipid profile, patients with NAFLD had increased ethanol levels, systemic and intestinal inflammation markers, and intestinal permeability when compared to the healthy children; (ii) synbiotic supplementation in addition to lifestyle changes decreased liver enzymes and improved fatty liver in approximately two-third of the patients; (iii) anthropometric parameters related to obesity decreased after synbiotic supplementation in addition to lifestyle changes; (iv) synbiotic supplementation improved TC and LDL levels, inflammatory markers, and ethanol levels; (v) mild elevation of ALT might be a marker of response to the synbiotic supplementation; and (vi) decrease in LDL levels and inflammation (CRP levels) might be effective in the improvement of fatty liver in addition to decrease in BMI.

In previous studies, it has been shown that obese patients with NAFLD had altered intestinal microbiota and intestinal barrier dysfunction due to environmental factors (high-fat/fructose diet) that cause low-grade systemic inflammation and metabolic endotoxemia (4). Circulating zonulin concentration was shown to increase in children and adolescents with NAFLD and correlated to the severity of hepatic steatosis (12). Additionally, other biomarkers of intestinal barrier dysfunction, such as occludin and tight junction (TJ) proteins, were also shown to increase in patients with NAFLD (5,12,13). In our study, we did not find any correlation to the severity of steatosis assessed by liver ultrasound; however, we found that it was correlated with intestinal inflammation markers. Similar results were found by Zak-Gołąb A et al. (13); zonulin concentration was correlated with intestinal inflammation and systemic microinflammation in obese subjects.

Ethanol is one of the major hepatotoxic microbiota-associated metabolite that is delivered to the portal circulation; moreover, it contributes to the accumulation of triglyceride and formation of reactive oxygen species in the liver in patients with altered intestinal microbiota (14). Increased levels of ethanol have been detected in animal and human studies with NAFLD (15). Zhu and colleagues compared NAFLD patients with obese and healthy controls and found that patients with NAFLD had significantly elevated ethanol levels compared to obese children without NAFLD and healthy controls. They showed that the difference was associated with the difference in the diversity of the microbiota between the groups; patients with NAFLD had ethanol-producer-dominant microbiota, including *Proteobacteria/Enterobacteriaceae/Escherichia*, compared to obese children without NAFLD and healthy controls (16). We found that the levels of ethanol were correlated to the TNF-α and ALT levels, suggesting the role of ethanol in the secretion of inflammatory cytokines (TNF-α) and progression of inflammation in the liver (17).

In our study, we found that systemic and intestinal inflammation markers are increased in children with NAFLD. In a recent study, the levels of pro-inflammatory cytokines and endotoxin-related parameters were shown to increase in patients with NAFLD, and pro-inflammatory cytokine levels were correlated with the histological severity of NAFLD (18). Fecal calprotectin levels were studied in obese children, and a correlation was found with BMI and abnormal oral glucose tolerance test in children (19).

The effects of probiotics and synbiotics on NAFLD have been studied previously. Vajro et al. (20) randomized 20 children with NAFLD for probiotic supplementation and placebo for 8 weeks and found that liver enzymes and serum antibodies to antipeptidoglycan-polysaccharide polymers were decreased in the probiotic-supplemented group compared to the placebo group at the end of 8 weeks. They concluded that probiotic supplementation may be used as a therapeutic tool in obese children with NAFLD who were non-compliant to lifestyle modifications. Alisi et al. (21) studied the effect of VSL#3 supplementation (450 billion colonies/sachet of viable, lyophilized bifidobacteria, lactobacilli, and Streptococcus thermophilus) on NAFLD in children. They found that 4 months of VSL#3 supplementation improved fatty liver and BMI in children by increasing the glucagon-like peptide 1 levels. Famouri et al. (22) analyzed the effect of a probiotic capsule (mixture of 4 probiotics strain) for 12 weeks in children with NAFLD and found an improvement on the sonographic grade of fatty liver and lipid profile at the end of 12 weeks.

The effects of synbiotic supplementation in patients with NAFLD was studied in adults; it was shown that 28 weeks of synbiotic supplementation (7 strains probiotic and fructooligosaccharide) in addition to lifestyle modifications was superior to lifestyle modifications alone for the treatment of NAFLD. Additionally, they showed that synbiotic supplementation attenuates inflammatory markers and decreases BMI and waist-to-hip ratio that was evident at week 14 and sustained until the end of the treatment (23). Similar results were found by Malaguarnera et al. (24) in a study on adults; *Bifidobacterium longum* with fructooligosaccharide and lifestyle modification for 24 weeks significantly reduces steatosis and the non-alcoholic steatohepatitis (NASH) activity index when compared to lifestyle modification alone. In our study, we found that 4 months of synbiotic supplementation in addition to lifestyle changes decreases the sonographic grade of fatty liver in approximately two-thirds of the children.

The effect of probiotic supplementation in patients with NAFLD was analyzed in a meta-analysis, and it was revealed that in addition to improvement in the grade of fatty liver, probiotics reduce liver enzymes, TC, TNF-α, and CRP and improve insulin resistance (25). Effects on BMI and other anthropometric parameters were not evident in adult patients, whereas it was more evident in children (26). Similar results were found in our study, except in improvement in insulin resistance. Additionally, we found that synbiotic supplementation reduces
LDL levels. Safavi et al. (27) showed a significant difference in all lipid parameters after 8 weeks of synbiotic supplementation in obese children. In contrast, Ipar et al. (28) found significant improvement only in TC and LDL levels and not in TG levels after synbiotic supplementation, as in our study. Effects of probiotics or synbiotics on TG levels have some controversies; Gao et al. (26) revealed that lipid-lowering effect of probiotics or symbiotics may vary in different countries/ethnicities. Additionally, types of the strain in probiotics, types of the prebiotics used for the study, and the duration of the treatment may affect lipid-lowering property (29,30). We found that improvement in lipid parameters; particularly in LDL levels, are important for the improvement of fatty liver in symbiotic-supplemented children. High LDL levels are a risk factor for the cardiovascular disease in patients with NAFLD (30). Decrease in LDL levels with the synbiotic supplementation will improve both fatty liver and future cardiovascular complications in children with NAFLD.

The limitations of our study were (i) the lack of liver biopsy for the assessment of the severity of steatosis and fibrosis. Since the participants were obese and did not have any serious clinical hepatic condition, we did not consider liver biopsy due to its invasive nature and limited the examinations to sonography and biochemical parameters; (ii) lack of microbiota analysis and (iii) a randomized control group limited the study; however, it was considered unethical to prescribe long-term “placebo plus lifestyle changes” for grade II-III fatty liver patients. It was shown that adherence to lifestyle modifications is poor in children; therefore, “placebo plus lifestyle changes” may worsen the steatosis if the patients do not adhere to the recommendations (31,32). The main strengths of our study were its novelty in the pediatric age group and that we used detailed biochemical analysis.

In conclusion, we showed that synbiotic supplementation in addition to lifestyle changes is effective in children with NAFLD. As shown in our study and previous studies, there is no suspicion for the positive effects of probiotics or synbiotics that added to lifestyle changes on NAFLD. As a consequence of our study, further prospective long-term studies are needed to analyze effect of probiotics or synbiotics on obesity-related cardiovascular complications, such as atherosclerosis and stroke.

Ethics Committee Approval: Ethics committee approval was received for this study from ethics committee of Karadeniz Technical University School of Medicine (Decision No: 2014/97).

Informed Consent: Informed consent was obtained from parents of the patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - M.C., A.A.I., E.S., G.K.; Design - M.C., A.A.I., E.S., G.K.; Supervision - M.C., A.A.I., E.S., G.K.; Resource - M.C., A.A.I., E.S.; Materials - M.C., A.A.I., E.S.; Data Collection and/or Processing - M.C., A.A.I., E.E., A.O., E.S.; Analysis and/or Interpretation- E.E., A.O., T.M.S., N.K.; Literature Search - M.C., A.A.I., E.S.; Writing - M.C., A.A.I., E.S., G.K.; Critical Reviews - M.C., A.A.I., E.S., G.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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