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Effects of Probiotics on Non-Alcoholic Fatty Liver Disease in Obese Children and Adolescents: A Randomized Clinical Trial

Running title: Probiotics and pediatric NAFLD

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Abstract

Objectives: This trial aims to evaluate the effects of some probiotics on sonographic and biochemical non-alcoholic fatty liver disease (NAFLD).

Methods: This randomized triple-blind trial was conducted among 64 obese children with sonographic NAFLD. They were randomly allocated to receive probiotic capsule (containing Lactobacillus acidophilus ATCC B3208, $3x10^9$ colony forming units, CFU; Bifidobacterium lactis DSMZ 32269, 6 x 10^9 CFU; B bifidum ATCC SD6576, $2x10^9$ CFU; L rhamnosus DSMZ 21690, $2x10^9$ CFU) or placebo for 12 weeks.

Results: After intervention, in the probiotic group the mean levels of alanine aminotransferase (ALT) decreased from 32.8 (19.6) to 23.1(9.9) U/L (P=0.02); and mean aspartate aminotransferase (AST) decreased from 32.2 (15.7) to 24.3 (7.7) U/L (P=0.02). Likewise the mean cholesterol, LDL-C, and triglycerides, as well as waist circumference decreased in the intervention group, without significant change in weight, BMI, and BMI Z-score. After the trial, normal liver sonography was reported in 17 (53.1%) and 5 (16.5%) of patients in the intervention and placebo groups, respectively.

Conclusions: The current findings suggest that a course of the abovementioned probiotic compound can be effective in improving pediatric NAFLD.

Key words: Non-alcoholic fatty liver disease, probiotics, obesity, children, adolescents

What is Known:

- Nonalcoholic fatty liver disease (NAFLD) is a growing disorder in children and adolescents.
- Low compliance of children for lifestyle change necessitates other treatments.
- No medical treatment is approved for pediatric NAFLD.

What is New:

- A course of probiotics studied had significant effect on improving the level of liver enzymes and sonographic fatty liver.
- A 12-week course of probiotics studied was effective in improving NAFLD and lipid profile of obese children
- The effects of the probiotics studied on improving pediatric NAFLD were independent of the weight status.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is considered as the most common chronic liver disease among children and adolescents (1). The condition is the hepatic manifestation of metabolic syndrome (2). NAFLD is a general term that refers to a spectrum of liver disease ranging from steatosis to non-alcoholic steatohepatitis (NASH) and fibrosis (3).

During last decades, the prevalence of NAFLD had a considerable escalating trend in the pediatric population; this increasing prevalence is mainly because of the nutritional transition and the rapid increase in the prevalence of childhood obesity (4). Epidemiological studies, using non-invasive diagnostic methods, have reported an estimated prevalence rate of NAFLD from 3-10% among general pediatric population (5) to 70-80% among obese children and adolescents (6).

The pathogenesis of pediatric NAFLD remains to be determined; and the condition is considered a challenging issue for both pediatricians and health care providers. It is documented that the complex interaction between genetic, epigenetic, and environmental factors results in the development and progression of NAFLD in children and adolescents (7).

It is also suggested that as an environmental factor, the gut microbiota have a significant impact on intra-hepatic fat accumulation; however, the mechanisms are not yet fully determined (8). The interaction between the liver and gut, i.e. the gut- liver axis, could explain the beneficial role of gut microbiota composition for pediatric NAFLD (9). Therefore, recent evidence suggests that targeting the axis using probiotics may be an appropriate approach for treating NAFLD (10, 11).

Lifestyle interventions are the first line treatment and prevention strategies for management of obesity and NAFLD. Given the poor compliance with lifestyle modification, some studies indicate that combination of these interventions with pharmacological interventions and dietary supplementation would have more favorable effect (12). It seems that among a recommended

series of therapeutic dietary supplements, probiotics might be the most promising agents because of their safety, tolerability, and efficacy (12).

Given the increasing trend of NAFLD and its related morbidities, inappropriate compliance of children for lifestyle interventions, lack of effective and safe medication, as well as growing recommendations to evaluate the effectiveness of probiotics through interventional studies, this study aims to evaluate the effects of probiotics on NAFLD in obese children and adolescents.

Methods

This randomized triple blind placebo controlled trial was conducted in 2014 in Isfahan, Iran. The eligibility criteria consisted of being aged 10-18 years and having age- and gender-specific body mass index (BMI) of >= 85 th percentile (13), along with sonographic findings of NAFLD; those with any evidence of other chronic liver diseases, history of alcohol drinking or chronic medication use were not included. For all participants, we excluded other causes of chronic hepatic disease including Wilson disease, autoimmune hepatitis, viral hepatitis (B and C) ,HIV, alfa 1 antitrypsin deficiency, hemochromatosis, and other metabolic diseases. Exclusion criteria were considered as: allergy and intolerance to the medication, and or loss of follow up.

Participants were randomly selected from children and adolescents referred to the pediatric clinics affiliated to Isfahan University of Medical Sciences (IUMS). Considering a power of 80% and type I error of 5%, we required a sample size of thirty-two participants in each group of medication and placebo to detect significant differences in liver ultrasound findings between the two groups.

The study protocol was reviewed by the Pediatrics Review Board of IUMS and approved by

Regional Ethics Committee of IUMS (research project number: 393891); it was registered at the Iranian registry of clinical trials (http://www. irct.ir; identifier: IRCT2013100414882N1). Oral assent, and written informed consents obtained from the participants and their parents.

All participants were referred to a radiologist for performing liver ultrasonography. Fatty liver was diagnosed based on the ultrasonographic diagnostic criteria (14-16).

The CONSORT diagram of the trial is presented in Supplemental Digital Content 1, Checklist, *http://links.lww.com/MPG/A820*. After including all eligible subjects to the study, by using computer-generated random numbers, the subjects were randomly assigned to one of the two groups receiving medication and placebo. Random allocation of patients to two groups was performed by sequentially numbered containers. An assistant performed randomization, so the group allocation was blinded for the investigators and participants.

For subjects in the medication group, one daily probiotic capsule was administered for 12 weeks. This medication (Prokid) was produced by Gostaresh Milad Pouya Company, Iran. The microbial strains were as follows: Lactobacillus acidophilus ATCC B3208, $3x10^9$ colony forming units, CFU; Bifidobacterium lactis DSMZ 32269, 6×10^9 CFU; B bifidum ATCC SD6576, $2x10^9$ CFU; L rhamnosus DSMZ 21690, $2x10^9$ CFU.

We used this medication, because in our clinical experience it had beneficial effects for other gastrointestinal problems, and also because it was available in Iran. Those in the placebo group received a daily placebo capsule (supplied by Amin Pharmaceutical Company) for 12 weeks. The probiotic and placebo capsules were identical in shape, size, and color; they were provided to participants with similar boxes. Neither the investigators nor the participants could distinguish the boxes of placebo from medication.

Healthy lifestyle habits were recommended for participants of both groups; they were encouraged to increase their daily activity, to walk fast, and to decrease the screen time, as well as to improve their dietary habits by increasing the intake of fruits and vegetables, and by decreasing the consumption of fast foods, high-fat meals, and sweet snacks.

All participants were checked each month for their weight, height, and their compliance to drug ingestion and for prescribing the placebo or the medication.

At the beginning and end of the trial, anthropometric examination, laboratory measurements, and ultrasonographic evaluation were performed for all participants. The within-group and between-group differences in the concentration of liver enzymes, grade of fatty liver, and anthropometric measures were compared before and after the trial.

Anthropometric measurements

Anthropometric measurements were performed by a trained nurse using standard protocols and calibrated instruments. Weight and height of participants were measured with light clothes and without shoes. BMI was calculated as weight (kg) divided by height squared (m^2). Waist circumference (WC) was measured by a non-elastic tape at a point midway between the lower border of the rib cage and the iliac crest at the end of normal expiration.

Laboratory measurements

Fasting venous blood sample was taken from the antecubital vein after 12 hours of fasting in the referral laboratory. Serum alanine aminotranferease (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase were measured on fresh samples by standard kits (Pars Azmoun, Tehran, Iran) using auto-analyzer (Hitachi, Japan).

Ultrasongraphic evaluation

An expert radiologist performed the ultrasonographic assessment before and after the trial using an ultrasound multi-frequency curvilinear 3.5-5 MHZ probe of Siemens Company (Sonoline G50 series, model number 7474922).

The ultrasonic detection of fat was used as a proxy measure. Grade I, II, and III were defined when the echogenicity of liver obscured the walls of portal vein branches, the diaphragmatic outline, and fatty infiltration. The following categories were used: (i) mild (grade1) minimal diffuse increase in hepatic echogenicity with normal visualization of diaphragm and intrahepatic vessel borders, (ii) moderate (grade 2) moderate diffuse increase in hepatic echogenicity with slightly impaired visualization of diaphragm and intrahepatic vessel borders, (iii) severe (grade 3) marked increase in hepatic echogenicity with poor penetration of posterior segment of right lobe of liver and poor or no visualization of hepatic vessels and diaphragm (17).

Statistical analysis

Statistical analysis was performed by SPSS version 20 (SPSS Inc., Chicago, IL, U.S.A.) using Student's *t*-test, Wilcoxon Signed Ranks, and Mann Whitney-U tests. P value of less than 0.05 was considered as statistically significant.

Results

Initially, 68 patients were enrolled in the study; 64 patients with NAFLD were selected and allocated into two intervention (n=32) and placebo (n=32) groups (Supplemental Digital Content 2, Figure 1, *http://links.lww.com/MPG/A821*). Baseline characteristics of patients in the two study groups are presented in Table 1. The groups were similar regarding age, gender, anthropometric characteristics, biochemical measurements, and radiologic findings of NAFLD.

The mean BMI level of all participants was greater than the 95th percentile, based on CDC curves (13).

The within group and between group changes after the trial are presented in Table2. Within group comparison showed significant decrease in mean levels of ALT, AST, and WC in the group receiving the probiotic medication. ALT and WC did not show significant changes in the placebo group. No significant change was seen for weight, BMI, and BMI Z-score at the end of trial.

In the intervention group 17 (53.1%) patients had normal liver sonography after the trial (P<0.001), whereas this frequency was 5 (16.5 %) in the placebo group (P=0.008). At the end of the trial, the intervention group had significantly lower mean level of liver enzymes than the interventional group. After the trial, compared to the placebo group, the frequency of normal liver ultrasonography was significantly higher, and the frequency of high grade fatty liver was significantly lower than the placebo group (P<0.05). Reduction in sonographic grading of NAFLD was in parallel to the decrease in mean level of liver enzymes (Table 2).

At the beginning of study, in the intervention group, 14 patients had abnormal ALT and AST; at the end of the trial, five patients had abnormal ALT and AST (78% improved, P= 0.004). ALT became normal in nine participants; seven out of them also had decreased sonographic grading of NAFLD (77.8%).

At baseline, the triglyceride and LDL levels had no significant difference in the intervention and placebo groups, but the cholesterol level was significantly higher in the intervention group. At the end of the trial, the mean serum level of cholesterol, triglyceride, and LDL decreased significantly in the intervention group (P< 0.001); whereas in the placebo group, the mean triglyceride level had significant decrease (P< 0.001).

Discussion

In this randomized controlled trial, we investigated the effect of kind of probiotics on weight status, ultrasonographic, and biochemical variables in obese children and adolescents with NAFLD. Our findings indicated that this supplement had some improving effect on the study variables, notably on liver enzymes and NAFLD sonographic grades.

Recent evidences have demonstrated that gut microbiota contribute to the development of NAFLD through the gut–liver axis. Our findings are consistent with some review studies, which evaluated the effect of probiotics on treatment of NAFLD through interventional studies, and have confirmed their beneficial effects (18, 19).

Though several animal and experimental studies have demonstrated improving effects of probiotics on different metabolic components of NAFLD, but available clinical trials on human population, especially among pediatric population, are scarce (19). In this regard, two previous clinical trials have investigated different strains of probiotics among children and adolescents (20, 21).

In a double blind clinical trial, Vajro et al. studied the effect of Lactobacillus rhamnosus strain GG in 20 obesity-related cases of pediatric NAFLD. They found that after 8 weeks, this strain resulted in normalization of fatty liver in 80% of cases and reduction in serum ALT level. They concluded that this probiotic could be used as an appropriate therapeutic tool for the treatment of children with NAFLD who were non-compliant with the recommended lifestyle modifications (20). Another clinical trial was conducted by Alisi et al. among 44 obese children with biopsy-proven NAFLD, they investigated the impact of VSL#3 vs. placebo in a 4-month trial. VSL#3 containing eight probiotic strains including **streptococcus thermophilus**, bifidobacteria ***(B.***

breve, B. infantis, and B. longum), lactobacillus acidophilus, L. plantarum, L. paracasei, and **L. delbrueckii** *subsp.* **Bulgaricus had significant improving effects on fatty liver and BMI (21).**

Some review studies have reported that probiotics could have a proper effect on improving fatty liver and reducing its grade from severe stage to mild or normal (20-22). In this trial, though we observed some improvement both in the intervention and placebo groups regarding the ultrasonographic grading of NAFLD, but the effects of the administered probiotic were better than placebo. The rate of improvement was 53% and 16.5% in the intervention and placebo groups, respectively. Our findings are consistent with the abovementioned trials conducted in the pediatric age group (20, 21).

It is well established that serum AST and ALT levels are clinical markers of liver damage (23). Some clinical trials that used probiotics have demonstrated that these agents may decrease the level of at least one of these markers (22, 24). The probiotic used in the current study reduced the concentrations of AST and ALT. After the trial, the levels of both markers were significantly lower in the probiotic group than in the placebo group. Improving ultrasonographic grading of NAFLD was also in parallel to the decreased liver enzymes.

Controversial results exist regarding the impact of probiotics on BMI and WC; some studies indicated beneficial effect, whereas others did not confirm it (20, 21, 25,26). The probiotic compound used in the current study had significant effect on WC, but not on weight and BMI. Lipid lowering effect of probiotics is reported both in animal and human studies (27-29).

The probiotic used in the current trial had some favorable effects on reducing the concentrations of cholesterol, LDL, and triglyceride. However, our finding on HDL is in line with some previous studies that did not document beneficial effects of probiotics in increasing this

lipoprotein with useful functions . Malaguarnera et al. suggested that HDL level m ight increase after long-term treatment of probiotics (21).

The probiotic used in this tria l resulted in som e improvement in the lipid prof ile of participants. At baseline, triglyc erides and LDL concentratio ns were not significantly different between the intervention and placebo groups, but total choles terol level was significantly higher in the intervention group. After the trial, total cholesterol, triglycerides, and LDL had m ild to moderate decrease in the group receivi ng the probiotic com pound. Triglycerides level had significant decrease in the placebo group; this change m ight be becau se of recomm endations on lifestyle change.

Study limitations and strengths: Small sample size, short durati on of trial and follow up, as well as lack of assessm ent of infla mmatory factor s are the m ain lim itations of our study. As the sample size was not large enough, we could not eval uate gender differences regarding the impact of probiotic. In addition, the probiotic used in th is trial was a m ixture of four probiotic strains. For obtaining more accurate resu lts, we suggest to design further studies in a way to evaluate a single probiotic agent and to conduct a crossover trial (including a washout period) to assess the gut microbiota. Another lim itation of the study is th at we did not consider liver biopsy because of its invasive nature; as the m ain problem of the study participants was only excess weigh, and they had no serious hepatic problem , we limited the exam inations to sonography and biochemical tests. The main strength of our study was its novelty in the pediatric age group.

Conclusion

A lim ited course of the probiotic adm inistered in th is trial cou ld be ef fective in im proving surrogate m arkers of NAFLD and the lip id p rofile. Further studies on va rious pro biotic s trains are recommended to be conducted in larger sample size and longer duration

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Table 1. Baseline demographic and anthropometric characteristics in patients with non-alcoholic fatty liver disease (NAFLD)

Variables P	robiotic group n=32	Placebo group n=32	P-value
Age Mean(SD)	12.7(2.2) 12.6	(1.7)	0.80
Gender (Boys) (%)	43.8 56.2		0.32
Weight (Kg)	61.6(20) 59.1	(13.6)	0.56
BMI (Kg/m^2)	26.44(4.3) 26.6	1(2.26)	0.85
BMI Z-score	2.87 2.91		0.69
WC (Cm)	82.2(14.7) 81.4	(6.8)	0.78
ALT (U/L)	32.8(19.6) 28.7	(13.7)	0.34
AST (U/L)	32.2(15.7) 30.2	(12.9)	0.57
Fatty liver (grade)			
- Grade I n(%)	20(62.5)	18(56.2)	0.61
- Grade II n(%)	12(37.5)	14(43.8)	

BMI; body mass index,WC; waist circumference, ALT; alanine aminotransferase, AST; aspartate aminotransferase

Table2. Mean level of waist circumference, liver enzymes and frequency of different grades of Non-alcoholic fatty liver disease (NAFLD) according to ultrasonographic findings, before and after trial in intervention and placebo groups

Variables	**Probiotic**			**Placebo**		
	Before	After	P value*	Before	After	P value*
WC(cm)	82.2(14.7) 8	0.3(15.1)	0.001	81.4(6.8)	80(7.2)	0.06
AST (U/L)	32.2(15.7)	24.3(7.7)**	0.002	30.2(12.9)	26.6(11.8)**	0.006
ALT(U/L)	32.8(19.6) 2	3.1(9.6)**	0.002	28.9(13.7)	26.2(12.9)**	0.20
Lipid profile						
-Cholestrol	157.31(57.11)£	145.06(47.87)	<0.001	108.00(24.33)£	105.43(23.56)**	0.07
-HDL-C	46.25(12.08)£	**	0.18	34.53(4.64)£	36.53(10.21)**	0.25
-LDL-C	87.93(28.74)	46.75(11.32)	<0.001	79.25(14.18)	78.31(12.93)	<0.001
-Triglyceride	112.53(50.46)	81.65(23.49) 100.56(44.80)	<0.001	96.03(20.65)	91.87(19.14)	<0.001
Fatty liver (grade)[n(%)]						
-Normal	0(0%)	17(53.1%) **		0(0%)	5 (16.5%) **	
- Grade I	20(62.5%)	8(25%)	<0.001	18 (56.2%)	15 (46.9%)	0.008
- Grade II	12(37.5%)	7 (21.9%)		14 (43.8%)	12 (37.5%)	

WC; waist circumference, ALT; alanine aminotransferase, AST; aspartate aminotransferase
* P-value for difference within groups throughout the study
£ P value<0.05 for difference between probiotic and placebo groups before intervention
**P value<0.05 for difference between probiotic and placebo groups after intervention