

## Effects of Multi-species Synbiotic Supplementation on Circulating miR-27a, miR-33a Levels, and Lipid Parameters in Adult Men with Dyslipidemia; A Randomized, Double-Blind, Placebo-Controlled Clinical Trial

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**Abstract**

MicroRNAs (miRNAs) have emerged as important regulators of lipid metabolism. Recent studies have suggested synbiotics may modulate miRNA expression and lipid metabolism. This study aimed to investigate the effects of synbiotic supplementation on circulating miR-27a, miR-33a, and lipid parameters in patients with dyslipidemia. Fifty-six eligible participants were randomly allocated to receive either synbiotic or placebo sachets twice a day for 12 weeks. Each synbiotic sachet contained  $3 \times 10^{10}$  CFU six species of probiotic microorganisms and 5 grams of inulin and fructooligosaccharide (FOS) as prebiotics. Serum miR-27a and miR-33a expression levels, serum lipids, and apolipoproteins, the fecal concentration of short-chain fatty acids (SCFAs), and *Firmicutes* and *Bacteroidetes* phyla were assessed before and after the study. Real-time PCR was used to determine the relative expression levels of miRNAs. The results showed synbiotic supplementation significantly downregulated the expression levels of miR-27a and miR-33a compared to the placebo group ( $p = 0.008$  and  $p = 0.001$ , respectively). Furthermore, the intervention group exhibited significant improvements in serum high-density lipoprotein (HDL-C), small dense low-density lipoprotein (sdLDL-C), apoA-I, and apoB-100 ( $p = 0.008$ ,  $p = 0.006$ ,  $p = 0.003$ ,  $p = 0.001$ , respectively). The results showed a significant negative correlation between miR-33a expression levels with HDL-C, butyrate, propionate, and a significant positive correlation with total cholesterol (TC), low-density lipoprotein (LDL-C), and sdLDL-C in the intervention group. Fecal bacteria and SCFAs were significantly increased in the intervention group. This study provides evidence that synbiotic supplementation can modulate miR-27a and miR-33a expression and improve lipid metabolism in patients with dyslipidemia.

**Keywords:** Synbiotic, Dyslipidemia, MicroRNA, Gut microbiota, Lipid profile

## Introduction

Dyslipidemia, a prevalent metabolic disorder and a major risk factor for Cardiovascular diseases (CVDs), is characterized by abnormal fasting blood lipid profile components including elevated TC levels greater than 200mg/dl, LDL-C levels exceeding 130mg/dl, triglyceride (TG) levels surpassing 150mg/dl, and decreased HDL-C levels below 40mg/dl in men and below 50mg/dl in women <sup>(1)</sup>. The prevalence rates are 39.7% for hypertriglyceridemia, 21.2% for hypercholesterolemia, 16.4% for increased LDL-C, 68.4% for low HDL-C, and 81.0% for dyslipidemia. Hypercholesterolemia and low HDL-C are more prevalent among women, while hypertriglyceridemia is more prevalent among men. Dyslipidemia is more prevalent in women (odds ratio of 1.8) <sup>(2)</sup>.

MiRNAs are small, non-coding RNA molecules, that play a pivotal role in the regulation of gene expression at the post-transcriptional level <sup>(3)</sup>. They regulate over 60% of human coding genes <sup>(4)</sup>. Dysregulation of miRNA expression has been implicated in numerous diseases, including cancer, CVDs, and metabolic conditions like hyperlipidemia <sup>(5)</sup>. Circulating miRNAs have emerged as potential biomarkers for various conditions, reflecting changes in cellular processes <sup>(6)</sup> and they can be used as an early diagnostic tool along with potential therapeutic properties <sup>(7)</sup>.

miRNAs have emerged as key regulators of lipid metabolism, influencing processes such as cholesterol synthesis, lipid transport, and adipocyte differentiation <sup>(5; 8; 9)</sup>. Among these, miR-27a and miR-33a have garnered significant attention for their roles in modulating genes involved in lipid homeostasis <sup>(10; 11)</sup>. miR-27a has been suggested as a main regulator of cholesterol metabolism <sup>(12)</sup> and miR-33 is a potential biomarker for therapeutic goals owing to its appropriate response to dietary interventions <sup>(13)</sup>. By focusing on these miRNAs, we aimed to investigate their potential modulation by synbiotic supplementation and their impact on lipid parameters in patients with dyslipidemia.

Growing evidence indicates that miRNA expression is influenced by the gut microbiota (GM) <sup>(14; 15; 16)</sup>. The main mechanism of action primarily involves the metabolites produced by the GM, including lipopolysaccharide (LPS), SCFAs, and amyloid <sup>(14)</sup>. Recent research has highlighted a strong correlation between dyslipidemia and alterations in the composition of GM <sup>(17; 18)</sup>. Firmicutes and Bacteroidetes are considered to be the most abundant bacteria in the gut,

comprising up to 90% of the GM <sup>(19)</sup>. Previous studies have shown that alterations in the abundance of these phyla are linked to metabolic disturbances <sup>(20)</sup>, justifying our interest in evaluating them in the context of synbiotic supplementation.

Synbiotics, a combination of probiotics and prebiotics, have gained attention for their potential to modulate GM composition <sup>(21)</sup> and promote host metabolism <sup>(22)</sup>. In addition, it has been suggested synbiotics may impact miRNA expression <sup>(23)</sup> and might be a complementary therapeutic strategy to manage dyslipidemia <sup>(24)</sup>.

While previous studies have investigated the potential benefits of synbiotic supplementation on traditional lipid parameters, this research aims to delve deeper by investigating the specific influence of multi-species synbiotic supplementation on circulating miRNAs related to lipid metabolism including miR-27a and miR-33a. These miRNAs through regulating expression levels of genes involved in lipid metabolism including LDLR and ATP binding cassette subfamily A member 1 (ABCA1) at the post-transcriptional level play crucial roles in lipid metabolism and may serve as novel biomarkers for dyslipidemia <sup>(4; 12)</sup>.

The objective of the study is to provide valuable insights into the molecular mechanisms underlying the effects of synbiotic supplementation on dyslipidemia. By evaluating both traditional lipid parameters and miRNA expression, the research aims to contribute to a comprehensive understanding of the potential therapeutic impact of multi-species synbiotics in managing dyslipidemia.

## **Methods**

### *Ethics statements*

The study protocol was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences in accordance with the Declaration of Helsinki (Approval code: IR.AJUMS.REC.1400.581).

The trial was registered on February 2nd, 2022, in the Iranian Registry of Clinical Trials (registration reference: IRCT20180128038540N2).

Before the start of the study, written informed consent was obtained from each participant, and their privacy and confidentiality were ensured throughout the trial.

### *Study design and population*

This study was a 12-week randomized, double-blind, placebo-controlled, parallel-group clinical trial conducted between May 2022 and September 2022. Fifty-six adult men diagnosed with dyslipidemia were recruited from patients referred to the Nutrition Clinic in Mahshahr, Iran. Inclusion criteria included willingness to participate, adults aged up to 60 years with mixed dyslipidemia (with TG levels between 200-400 mg/dl and LDL-C levels between 130-160 mg/dl).

Exclusion criteria encompassed the use of chemical or herbal lipid-lowering drugs, presence of familial dyslipidemia, any history of heart, kidney, liver, gastrointestinal, endocrine, autoimmune diseases, malignancies, significant changes in body weight, diet or lifestyle in the last 6 months, smoking, alcohol consumption, drug abuse, intake of antibiotics or dietary supplements of antioxidants, probiotics, prebiotics, or synbiotics in the past 6 months, and frequent travel.

The participants' sociodemographic characteristics including age, gender, ethnicity, marital status, educational level, occupational status, and smoking, were assessed using a self-report sociodemographic questionnaire during the recruitment phase. Due to logistic limitations, this study specifically included male patients.

### *Intervention*

The synbiotic formula used in this study was developed based on previous research findings indicating its efficacy in modulating gut microbiota and lipid metabolism, which determined the composition and dosage of probiotics and prebiotics. The synbiotic supplement sachet contained  $3 \times 10^{10}$  CFU six lyophilized probiotic strains, including *Lactobacillus (L.) acidophilus* <sup>(25)</sup> ATCC4356, *L. fermentum* <sup>(26)</sup> DSM14241, *L. plantarum* <sup>(27)</sup> ATCC14917, *Bifidobacterium (B.) longum* <sup>(28)</sup> BAA-999, *B. lactis* <sup>(29)</sup> ATCC27536, and *Saccharomyces (S.) boulardii* <sup>(30)</sup> CNCM I-745. Inulin and FOS <sup>(24)</sup> were included as prebiotics at a dosage of 5 grams in equal amounts <sup>(31)</sup>. Placebo sachets contained 5 grams of corn starch, while the synbiotic and placebo sachets were identical in flavor, aroma, color, and appearance. They were produced and packaged by Faradaru Pharmaceutical Company in Tehran, Iran. Participants were instructed to dissolve the synbiotic or placebo powders in a cup of water (240 ml) and take them twice a day (BID), 30 minutes before lunch and dinner for 12 weeks. The study duration was based on the recommendation of a

systematic review and meta-analysis study on the effects of synbiotics on lipid profile <sup>(24)</sup>. The participants received two boxes of either the active ingredient or placebo every four weeks, each containing 30 sachets along with storage instructions below 25°C as recommended by the manufacturer.

#### *Randomization and blinding*

In this study, a simple randomization technique was implemented through computer-generated random numbers by a third party to randomly assign eligible individuals to two groups. The enrollment and screening procedures were carried out by a research assistant under the supervision of the primary investigator. To maintain blinding, both the patients and the researchers involved in the study were unaware of the assigned interventions. The synbiotic and placebo sachets, along with their packaging, were carefully designed to have similar color and appearance. Additionally, to ensure individual blinding, an independent person not associated with the study coded the packages, effectively concealing the identity of the interventions.

#### *Dietary intake and physical activity assessment*

The participants' nutrient and calorie intake were evaluated using a 3-day food record (two weekdays and one weekend day) at the beginning and end of the intervention. The dietary intake was analyzed using Nutritionist IV software (First Data Bank; Hearst Corp, San Bruno, CA, USA). To assess the participants' physical activity level (PAL), a self-reported questionnaire on PA <sup>(32)</sup> was administered at both baseline and end of the study. PA was determined using the values of the metabolic equivalent of task-hours (MET-h)/day for each PA, regarding the time spent in each activity. Throughout the study, participants were instructed to maintain their regular diet and PA patterns.

#### *Anthropometric measurements*

Anthropometric measures, including height, weight, waist circumference (WC), body fat percentage (BFP), visceral fat percentage (VFP), and Body Mass Index (BMI) were measured at the baseline and end of the trial. Height was measured using a digital stadiometer (InBody, BSM170, South Korea) with an accuracy of 0.1 cm, without shoes, head facing forward, and heels attached to the device. BFP, VFP, and weight (with an accuracy of 0.1 kg) were determined using an electrical body composition analyzer (InBody270, South Korea) with a light cloth and

no shoes. WC was measured with a non-flexible tape in the standing position from the midpoint between the lowest rib and iliac crest. BMI was calculated by dividing the weight in kilograms by the height squared in meters.

### *Laboratory assessment*

#### *Assessment of serum lipid parameters*

For biochemical assessment, 5ml of venous blood samples were collected after 10–12 h of overnight fasting at baseline and after 12 weeks of the study. The serum samples were separated by centrifugation and immediately frozen at  $-70^{\circ}\text{C}$  until analysis.

Serum levels of TC, TG, HDL-C, and very low-density lipoprotein (VLDL) were assessed using an enzymatic method (ParsAzmun Company, Tehran, Iran). The concentration of LDL-C was measured using the Friedewald formula<sup>(33)</sup>. The serum levels of sdLDL-C, apolipoproteins including apoA-I, and apoB-100 were assayed using enzyme-linked immunosorbent assay (ELISA) kits (Randox Company, Crumlin, United Kingdom).

#### *Analysis of circulating miR-27a and miR-33a*

To determine the expression levels of circulatory miR-27a and miR-33a, quantitative Real-time PCR was performed using the ABI StepOne Plus detection system (ABI, USA). The miRNA was extracted using miRcute Serum/Plasma miRNA Isolation Kit as recommended by the manufacturer's protocol (TIANGEN, China). The purity of RNA at 260/280 OD ratio and the RNA integrity were evaluated using an Eppendorf  $\mu\text{Cuvette G1.0}$  microvolume measuring cell (Eppendorf, Germany). High-purity RNA with an OD of 260/280 ratio above 1.8 was used for cDNA synthesis. The cDNA was synthesized using miRcute miRNA First-strand cDNA Synthesis Kit (TIANGEN, China) based on the polyadenylation method. miRNA levels were quantified by the qRT-PCR method using the miRcute miRNA qPCR Detection Kit (SYBR Green) (TIANGEN, China). The thermal program consisted of  $95^{\circ}\text{C}$  for 5 min, followed by 40 cycles of  $94^{\circ}\text{C}$  for 15 sec,  $60^{\circ}\text{C}$  for 15 sec, and  $72^{\circ}\text{C}$  for 20 sec. Each experiment was performed with two technical replicates. The relative expression level of the target genes was compared to the miR-16 gene as a housekeeping gene. Two separate reactions without cDNA or with RNA were performed in parallel as controls. Melting curve analysis was performed to verify the presence of gene-specific peaks and the absence of primer dimers. Relative quantification was

performed according to the comparative  $2^{-\Delta\Delta Ct}$  method, using cycle threshold (Ct) values extracted from StepOne software version 2.3. The miRNAs sequences (miR27a, GeneBank Accession No: NR\_029501.1; miR33a, GeneBank Accession No: NR\_029507.1, miR16: GeneBank Accession No: NR\_029486.1) were obtained from Gene Bank (<https://www.ncbi.nlm.nih.gov/nucleotide/>). Primers were designed using the sRNAprimerDB online tool (<http://www.srnprimerdb.com/submitA>). The sequence of primers and adapter were as follows: miR27a: 5'-GGCTTAGCTGCTTGTGA-3', miR33a: 5'-GTGCATTGTAGTTGCATTG-3', miR16: 5'-AGCAGCACGTAAATATTGG-3' and adapter sequence: 5'-GAACATGTCTGCGTATCTC-3'. PCR products were analyzed by 2% agarose gel electrophoresis to confirm the predicted size (approximately 90 bp, including mature miRNA and adapter sequences) of amplified miRNAs. Calculation of the amplification efficiency was determined from the slope of the standard curve as described previously<sup>(34)</sup>. Standard curves were generated by performing qRT-PCR on each gene using serially diluted cDNA samples. The amplification efficiency was then calculated using the formula  $E = (10^{-1/\text{slope}} - 1) \times 100\%$ .

#### *Assessment of fecal bacteria*

This study collected fresh stool samples from patients at the beginning and end of the study, which were stored at 4°C and then frozen at -80°C with an ID code for further analysis. The number of Firmicutes and Bacteroidetes populations was determined using real-time PCR analysis with the absolute quantification method. The PCR assay used a hydrolysis probe targeting the 16S rRNA gene and a standard curve was constructed using serially diluted plasmid DNA containing the 16S rDNA from standard bacterial species. DNA extraction was performed using the Stool DNA isolation kit (Cat No: FASTI 001-1, FavorPrep Stool DNA Isolation Mini Kit, FAVORGEN, Taiwan), and amplification and detection of DNA were performed with a YTA qPCR Probe MasterMix kit (Yekta Tajhiz, Iran) in an ABI StepOnePlus. The standard curves were generated using triplicate seven-fold dilutions of plasmid DNA from 0-10<sup>7</sup> plasmid/reaction, and the results were expressed as the LOG<sub>10</sub> colony forming unit (CFU)/gr of stool. The amplification efficiency was calculated using the formula  $E\% = (10^{-1/\text{slope}} - 1) \times 100$ . E is one if the amount of PCR product exactly doubles with each cycle and the efficiency expressed in percent is 100%.



*Assessment of fecal short-chain fatty acids*

Assessment of fecal SCFAs including acetate, butyrate, and propionate was performed by Gas Chromatography (GC) method using a GC Device (GC-2014 Shimadzu, apparatus, Japan) following established protocols <sup>(35)</sup>. Before analysis, samples were thawed and processed according to standardized procedures. Initially, a 500 mg sample of homogenized fecal matter was treated with a 15% aqueous azide solution in a Falcon tube. Subsequently, phosphoric acid was added to each 100 mg of the sample, ensuring complete homogenization. Following this, 2-ethyl butyraldehyde was introduced as an internal standard. After centrifugation, the supernatant was filtered for injection. The GC analysis was performed utilizing a Shimadzu GC-2014 apparatus with specific column and carrier gas settings. This methodological approach, which involved precise preparation steps and sophisticated GC instrumentation, facilitated the accurate quantification of SCFAs in fecal samples.

*Compliance*

To monitor patient compliance with the consumption of sachets, individuals were asked to return unconsumed sachets at the end of each month. The number of sachets remaining in boxes determined their compliance. At the end of the study, participants who consumed less than 90% of their sachets were excluded. To increase the compliance rate and decrease the dropout rate among the participants, daily messages were sent to their cell phones, and weekly phone calls were made to remind them to take the supplement and ask about possible side effects.

*Sample size*

The sample size was calculated using the equation for estimation of sample size in clinical trials <sup>(36)</sup> based on the parameter of total cholesterol in previous research <sup>(37)</sup> (with an alpha error of 0.05 and a beta error of 20%). Twenty-five participants per group were estimated and after considering an anticipated dropout rate of 10%, 56 individuals diagnosed with dyslipidemia (28 patients per group) were recruited.

### *Statistical analysis*

The Kolmogorov-Smirnov test was used to evaluate the normality of data. Between-group and within-group comparisons were conducted by independent sample t-test or paired sample t-test, respectively. A general linear model was used to control confounding variables. A Pearson's correlation coefficient analysis was used to describe the association between miRNA expression levels and other variables. Intention-to-treat (ITT) and per-protocol (PP) analyses were performed to analyze the data. An ITT analysis was conducted, addressing missing data through mean imputation, and encompassed all randomized participants, irrespective of protocol adherence or missing data. The PP analysis, which included only participants who completed the study, yielded consistent results, highlighting the robustness of our findings. Due to space constraints, only the results from the PP analysis are presented in this manuscript. The Statistical Package for Social Science software (SPSS Inc., Chicago, IL, USA, version 28) was used for the statistical analysis of data.  $p \leq 0.05$  was considered statistically significant.

### **1. Results**

The consort flow diagram is shown in Figure 1. Of the 105 volunteers enrolled in this study, 56 patients were eligible to participate and randomly assigned to the synbiotic (n = 28) and placebo (n = 28) groups. Six patients (3 patients from each group) were excluded from the study because of a low compliance rate. A high compliance rate (90.0%) was observed in the 50 patients who completed the study. All the recruited participants were male with an average age of 42.4 years.

Table 1 provides data on the anthropometric indices before and after the study for both the intervention and placebo groups. The study revealed no significant differences in these variables between the two groups at both baseline and post-intervention. Additionally, there were no significant changes observed in these parameters within each group at the study's conclusion compared to the baseline. Furthermore, the dietary intake and PAL of participants in both the intervention and placebo groups showed no significant differences between groups and no significant changes within each group.

Serum lipid parameters and apolipoproteins of the two groups are detailed in Table 2. At the baseline, no significant difference was observed between the two groups in these parameters.

The mean serum HDL-C and apoA-I concentration was significantly increased, and serum sdLDL-C and apoB-100 significantly decreased after 12 weeks of synbiotic supplementation in the intervention group (Table 2). However, no significant difference in the above parameters was observed in the placebo group at the end of the trial. TC, TG, LDL-C, and VLDL didn't have a significant change in both groups after intervention.

At the end of the study, miR-27a and miR-33a relative expression levels were significantly decreased in the intervention group compared with the placebo group ( $p = 0.008$ ,  $p = 0.001$ ) (Fig. 2).

The mean of fecal SCFAs including acetate, butyrate, and propionate significantly increased in the intervention group at the end of the study. No significant change was observed in the placebo group. (Table 3).

In the intervention group, the mean CFU of *Firmicutes* and *Bacteroidetes* was significantly increased ( $p < 0.0001$ ,  $p < 0.0001$ ) (Table 3).

A strong positive correlation was observed between miR-33a expression levels with serum TC, LDL-C, and sdLDL-C in the intervention group ( $r = 0.60$ ,  $p = 0.002$ ), ( $r = 0.66$ ,  $p < 0.001$ ), and ( $r = 0.68$ ,  $p < 0.001$ ) respectively, according to Pearson's correlation coefficient analysis. In addition, a negative correlation also existed between the changes in miR-33a expression levels with serum HDL-C ( $r = -0.49$ ,  $p = 0.012$ ) and fecal concentrations of butyrate and propionate ( $r = -0.51$ ,  $p = 0.010$ ;  $r = -0.49$ ,  $p = 0.014$  respectively) in the intervention group (Table 4).

## 2. Discussion

Research has demonstrated that GM-targeted interventions including synbiotic administration can influence miRNA expression patterns <sup>(38)</sup>. However, the specific impact of synbiotic supplementation on miRNAs associated with lipid metabolism in patients with dyslipidemia has remained elusive until now.

Our study represents the first investigation into the effects of synbiotic supplementation on the expression levels of miR-27a and miR-33a in dyslipidemia. We conducted a 12-week synbiotic supplementation trial and evaluated the impact of this intervention on circulating miRNA levels, serum lipid parameters, fecal SCFA concentrations, and most abundant gut bacteria.

Existing evidence on the effects of synbiotics on miRNA expression is limited. In an in-vivo study, administration of *L. rhamnosus* in mice decreased ethanol-elevated miR122a levels and attenuated ethanol-induced liver injury <sup>(39)</sup>. Rodríguez-Nogales et al. investigated the effects of two probiotic species, *L. fermentum* and *L. salivarius*, on inflammation-related miRNAs, demonstrating positive outcomes on gut dysbiosis and miRNA expression levels in male mice <sup>(40)</sup>. Conversely, a pilot study indicated that a 6-month supplementation with a multi-species probiotic did not alter the expression levels of miR-29a-c in HIV-positive subjects <sup>(41)</sup>.

Our results showed a significant downregulation of miR-27a and miR-33a expression levels following synbiotic supplementation. Downregulation of miR-33a through the increasing expression of ABCA1 and ABCG1 genes could improve reverse cholesterol transport leading to elevated circulating HDL-C levels <sup>(42)</sup>. Moreover, aligned with our results, Simionescu et al. found a significant positive correlation between miR-33a expression and TC, TG, LDL-C, and apoB-100 and an adverse correlation, but not statistically significant with HDL-C <sup>(43)</sup>.

Our findings further support the potential therapeutic implications of inhibiting miR-27a expression to alleviate hypercholesterolemia. This inhibition could enhance the expression of the LDLR (primary route for LDL-C clearance from circulation) gene and reduce LDLR degradation by lowering proprotein convertase subtilisin/kexin type 9 (PCSK9) levels <sup>(44)</sup>.

While our study primarily emphasized the regulation of miRNA expression, it is important to acknowledge that GM-targeted interventions may exert their metabolic effects through diverse mechanisms. For instance, the activation of peroxisome proliferator-activated receptors (PPARs), which play a pivotal role in lipid metabolism and are known to be influenced by GM composition and metabolites <sup>(45)</sup>.

Approximately 90% of the adult GM consists of the predominant phyla *Bacteroidetes* and *Firmicutes*. These phyla are essential for producing SCFAs that are suggested to mediate the several positive effects of synbiotics on lipid metabolism <sup>(46; 47)</sup>. SCFAs activate the adenosine monophosphate-activated protein kinase (AMPK) pathway, which inhibits the synthesis of fatty acids, cholesterol, and TG, potentially improving lipid disorders <sup>(48)</sup>. Particularly, butyrate may increase HDL-C levels by upregulating genes involved in HDL-C synthesis and transport, such as apoA-I and ABCA1 <sup>(49)</sup>. Additionally, SCFAs enhance gut barrier function, decreasing gut

permeability which in turn, can restrict the entry of bacterial endotoxins into the bloodstream, consequently improving low-grade inflammation and lipid metabolism <sup>(50)</sup>. The observed increase in fecal SCFA concentrations and alterations in gut bacteria abundance following synbiotic supplementation suggest a potential mechanistic link between GM modulation and lipid metabolism.

Although the current study did not observe significant reductions in serum TC and LDL-C levels, the observed improvements in the serum levels of apolipoproteins including apoA-I and apoB-100, and sdLDL-C underscore the importance of considering alternative lipid markers for assessing cardiovascular risk, notably, serum sdLDL-C levels have emerged as a more accurate predictor of cardiovascular risk than LDL-C levels, emphasizing the clinical relevance of our findings <sup>(51)</sup>. Moreover, serum apoB-100 which has shown a significant reduction in the current study is suggested to be a more useful and accurate biomarker than LDL-C in determining atherogenic lipid levels <sup>(52)</sup>.

There is a discrepancy in the effects of synbiotic supplementation on lipid parameters across different studies <sup>(37; 53; 54; 55)</sup> underscores the need for further research to elucidate the underlying mechanisms and address existing controversies. Factors such as subject characteristics, synbiotic composition, and study design and duration may contribute to variability in outcomes.

our study provides valuable insights into the molecular and microbial pathways underlying synbiotic-mediated improvements in lipid metabolism. By demonstrating significant alterations in miR-27a and miR-33a expression levels and gut bacteria abundance following synbiotic supplementation our findings contribute to a deeper understanding of the therapeutic potential of synbiotics in dyslipidemia management.

Further research exploring the long-term effects of synbiotic supplementation on GM composition, miRNA regulation, and lipid metabolism is warranted to fully elucidate its therapeutic potential and clinical implications.

## **Limitation and strength**

Our study has several strengths. First, this is the first randomized clinical trial to investigate the effects of synbiotic supplementation on circulating levels of miR-27a and miR-33a in patients with dyslipidemia, providing novel insights into the potential therapeutic benefits of synbiotics in this population. Furthermore, the components of the synbiotic supplement were determined based on prior research that indicated the efficacy of the specific probiotic species and prebiotic fibers used in the synbiotic formula for improving lipid metabolism. Additionally, the study was conducted among a homogenous group of non-smoker adult men with similar ethnicity and lifestyle, minimizing confounding factors. Lastly, the high compliance rate among participants who completed the 12-week study period in both groups strengthens the reliability of the results.

However, there are a few limitations to acknowledge. Firstly, the study included only male participants due to logistic constraints. Secondly, the study did not examine the target gene expression of the investigated miRNAs, which could be an area for future research to gain a deeper understanding of their functional implications.

## **Conclusions**

In conclusion, this study provides valuable insights into the effects of synbiotic supplementation on the expression of lipometabolic-related miRNAs and serum lipid biomarkers in patients with dyslipidemia. Additionally, the findings suggest that synbiotic supplementation may positively influence GM composition and SCFA production, which are known to impact lipid metabolism. Overall, this study's results support the potential of synbiotics consumption as a promising approach for modulating miRNA expression and improving lipid parameters in patients with dyslipidemia. Further studies with larger sample sizes and longer durations, including both sexes, are warranted to confirm and expand upon these findings.

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## **Author contribution**

All authors contributed to the design of the research. Sh.S. conducted the research, analyzed data, and wrote the manuscript. A.M. supervised the study. All authors contributed to review and edit the manuscript draft. All authors read and approved the final manuscript

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## **Declaration of interests**

The authors declare none.

**Abbreviations:** miRNA: microRNA, FOS: fructooligosaccharide, SCFAs: short chain fatty acids, HDL-C: high-density lipoprotein, sdLDL-C: small dense low-density lipoprotein, TC: total cholesterol, LDL-C: low-density lipoprotein, CVD: Cardiovascular diseases, TG: triglyceride, GM: gut microbiota, LPS: lipopolysaccharide, ABCA1: ATP binding cassette subfamily A member 1, PAL: physical activity level, MET: metabolic equivalent of task, WC: waist circumference, BFP: body fat percentage, VFP: visceral fat percentage, BMI: body mass index, VLDL: very low-density lipoprotein, Ct: cycle threshold, CFU: colony forming unit, GC: gas chromatography, ITT: intention to treat, PP: per protocol, PCSK9: proprotein convertase subtilisin/kexin type 9, PPARs: peroxisome proliferator-activated receptors, AMPK: AMP-activated protein kinase

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**Table 1.** Anthropometric measures before and after study between and within groups

Variables		Intervention (n=25)		Placebo (n=25)		<i>P</i> *
		Mean	SD	Mean	SD	
Weight (kg)	Before	90.8	16.5	87.3	10.6	0.37
	After	91.1	15.5	87.5	8.6	0.37
	<i>P</i> **	0.42		0.14		
BMI (kg/m <sup>2</sup> )	Before	30.1	4.9	28.7	4.1	0.30
	After	30.2	4.9	28.8	4.1	0.31
	<i>P</i> **	0.40		0.32		
WC (cm)	Before	104.3	10.7	101.7	8.8	0.36
	After	104.4	10.6	102.0	8.4	0.60
	<i>P</i> **	0.80		0.47		
BFP (%)	Before	32.9	6.5	31.1	5.6	0.29
	After	33.3	7.2	30.9	5.5	0.19
	<i>P</i> **	0.27		0.56		
VFP (%)	Before	13.04	4.4	11.4	3.6	0.16
	After	13.1	4.6	11.4	3.3	0.10
	<i>P</i> **	0.57		0.77		

BMI, Body Mass Index; WC, Waist Circumference; BFP, Body Fat Percentage; VFP, Visceral Fat Percentage

\* *P* for between-group comparison was reported based on an independent sample t-test

\*\* *P* for within-group comparison was reported based on a paired sample t-test

Data are presented as mean and SD

$p < 0.05$  is statistically significant

**Table 2.** Serum lipid parameters before and after intervention between and within groups

Variables		Intervention(n=25)		Placebo(n=25)		<i>P</i> <sup>*</sup>
		Mean	SD	Mean	SD	
TG (mg/dl)	before	234.0	57.3	228.6	59.9	0.7
	After	236.9	54.9	226.7	56.4	0.5
	<i>P</i> <sup>**</sup>	0.8		0.9		
TC (mg/dl)	before	242.9	16.3	242.2	24.6	0.90
	After	239.2	30.2	242.7	26.3	0.7
	<i>P</i> <sup>**</sup>	0.49		0.9		
LDL-C (mg/dl)	before	158.8	20.1	158.7	16.7	0.9
	After	153.8	26.1	160.3	26.1	0.4
	<i>P</i> <sup>**</sup>	0.4		0.7		
HDL-C (mg/dl)	before	40.8	6.6	39.7	9.0	0.6
	After	42.8	7.4	40.7	7.1	0.3
	<i>P</i> <sup>**</sup>	<b>0.008</b>		0.32		
VLDL (mg/dl)	before	42.8	14.0	43.2	9.9	0.9
	After	43.2	12.9	43.6	12.6	0.9
	<i>P</i> <sup>**</sup>	0.8		0.9		
SdLDL-C (mg/dl)	before	42.0	8.3	43.3	5.9	0.52
	After	37.2	9.6	42.2	8.4	0.2
	<i>P</i> <sup>**</sup>	<b>0.006</b>		0.3		
ApoA-I (mg/dl)	Before	134.0	17.6	137.2	17.7	0.5
	After	148.0	19.6	140.0	11.6	0.08
	<i>P</i> <sup>**</sup>	<b>0.003</b>		0.5		
ApoB-100 (mg/dl)	Before	119.9	18.8	114.8	18.1	0.3
	After	104.0	17.6	117.0	18.0	<b>0.013</b>
	<i>P</i> <sup>**</sup>	<b>&lt;0.001</b>		0.6		

TG, Triglyceride; TC, Total cholesterol; LDL-C, Low-density Lipoprotein cholesterol; HDL-C, High-density Lipoprotein cholesterol, VLDL, Very Low-density Lipoprotein; SdLDL-C, Small dense Low-density Lipoprotein cholesterol

\* *p* for between-group comparison was reported based on an independent sample t-test

\*\* *p* for within-group comparison was reported based on paired sample t-test

Data are presented as mean and SD

*p* < 0.05 is statistically significant

**Table 3.** Fecal SCFAs and bacteria before and after intervention between and within groups

Variable		Intervention (n = 25)		Placebo (n = 25)		P*
		Mean	SD	Mean	SD	
Acetate (mmol/gr)	before	13.39	2.70	13.41	2.56	0.98
	After	15.26	2.95	13.79	3.46	0.11
	<i>p</i> <sup>**</sup>	<b>&lt; 0.0001</b>		0.39		
Butyrate (mmol/gr)	before	2.43	0.66	2.67	1.04	0.34
	After	2.53	0.64	2.61	0.83	0.69
	<i>p</i> <sup>**</sup>	0.10		0.41		
Propionate (mmol/gr)	before	4.35	1.10	4.94	1.26	0.08
	After	5.20	1.17	5.03	0.96	0.59
	<i>p</i> <sup>**</sup>	<b>&lt; 0.0001</b>		0.65		
<i>Firmicutes</i> (CFU/gr)	before	5.78	0.71	5.76	0.89	0.92
	After	6.25	0.79	5.95	0.88	0.22
	<i>p</i> <sup>**</sup>	<b>&lt; 0.0001</b>		0.1		
<i>Bacteroidetes</i> (CFU/gr)	before	6.29	0.79	6.19	0.96	0.68
	After	6.81	0.78	6.22	0.78	<b>0.011</b>
	<i>p</i> <sup>**</sup>	<b>&lt; 0.0001</b>		0.90		

\* *p* for between-group comparison was reported based on an independent sample t-test

\*\* *p* for within-group comparison was reported based on paired sample t-test

Data are presented as mean and SD

*p* < 0.05 statistically significant

**Table 4.** Parametric Pearson's correlation coefficient of miR-27a and miR-33a expression levels with other variables changes in two groups

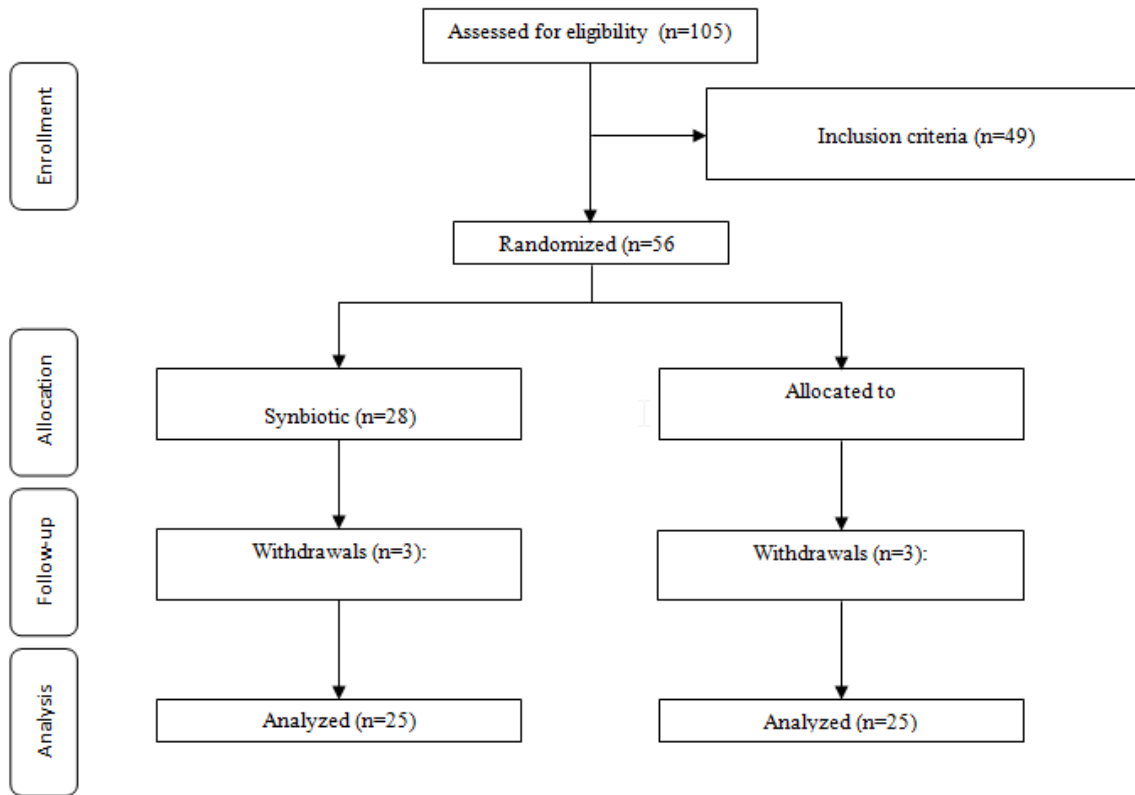
	<b>intervention</b>				<b>Placebo</b>			
	MiR-27a		MiR-33a		MiR-27a		MiR-33a	
	r	p	r	p	r	p	r	p
TC	0.20	0.35	0.60	<b>0.002</b>	0.13	0.54	0.39	0.051
TG	0.17	0.42	0.30	0.13	0.35	0.08	0.14	0.49
LDL-C	-0.05	0.83	0.66	<b>&lt;0.0001</b>	0.34	0.10	0.38	0.06
HDL-C	0.09	0.70	-0.49	<b>0.012</b>	0.14	0.54	-0.09	0.67
VLDL	0.18	0.40	0.38	0.06	-0.32	0.12	-0.09	0.66
sdLDL-C	0.10	0.65	0.68	<b>&lt;0.0001</b>	0.31	0.13	0.46	<b>0.021</b>
apoA-I	-0.39	0.05	0.017	0.93	-0.45	<b>0.023</b>	-0.13	0.6
apoB-100	0.026	0.9	-0.06	0.75	0.08	0.70	0.18	0.4
Acetate	-0.08	0.68	-0.29	0.16	-0.27	0.19	0.02	0.92
Butyrate	0.24	0.25	-0.51	<b>0.010</b>	-0.19	0.36	-0.15	0.46
Propionate	0.08	0.69	-0.49	<b>0.014</b>	-0.14	0.52	-0.16	0.44
Firmicutes	0.23	0.32	0.20	0.32	-0.05	0.81	0.24	0.28
Bacteroidetes	-0.06	0.80	0.23	0.39	-0.12	0.59	-0.17	0.42

TC, Total cholesterol; TG, Triglyceride LDL-C, Low-density Lipoprotein cholesterol; HDL-C, High-density Lipoprotein cholesterol VLDL, Very Low-density Lipoprotein; sdLDL-C, small dense Low-density Lipoprotein cholesterol.

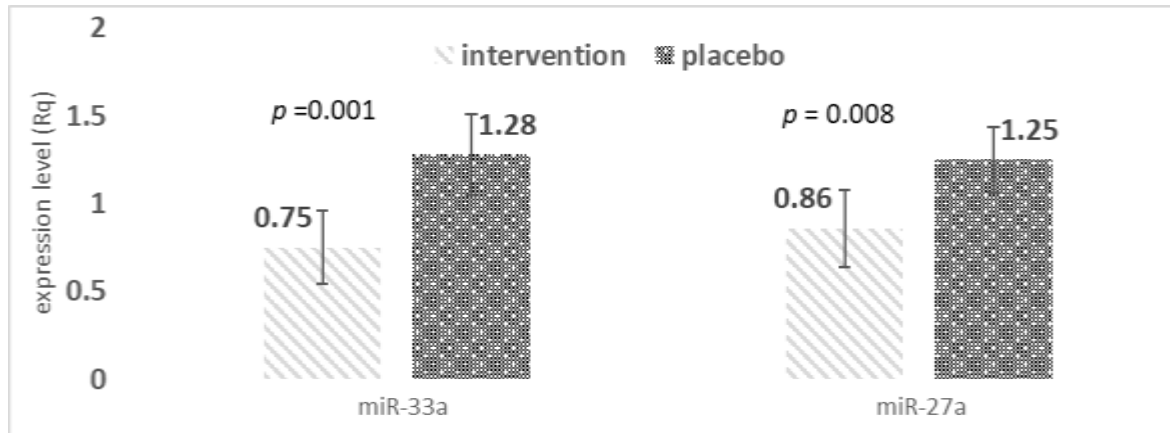
Statistical analysis was done using Pearson's Correlation Coefficient

$p < 0.05$  was considered statistically significant





**Fig. 1.** Consort flowchart



**Fig. 2.** miR-33a and miR-27a relative expression levels in the study groups

Each point represents a mean  $\pm$  CI 95%.

Data analysis was done using analysis of covariance (ANCOVA) adjusting age, dietary intake, PAL, and BMI