Effect of Probiotic Supplementation along with Calorie Restriction on Metabolic Endotoxemia, Trimethylamine-N-Oxide, Inflammation, Metabolic Factors, and Gut Microbiota Profile in Coronary Artery Disease Patients: A Double Blind Placebo Controlled Randomized Clinical Trial

CURRENT STATUS: POSTED

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DOI: 10.21203/rs.3.rs-17854/v1

SUBJECT AREAS
Endocrinology & Metabolism  Nutrition & Dietetics

KEYWORDS
Coronary Artery Disease, lipopolysaccharide, gut microbiota, Metabolic endotoxemia, Probiotic, Trimethylamine-N-oxide
Abstract
Background Dysbiosis has been associated to increased microbial translocation, leading to chronic inflammation in cardiovascular diseases (CVD). It has been proposed that modulation of gut microbiota by probiotic might modify metabolic endotoxemia. Therefore, the purpose of this study was to examine the effects of Lactobacillus rhamnosus GG (LGG) on metabolic endotoxemia, Trimethylamine-N-oxide (TMAO) and gut microbiota profiles in CVD subjects.

Methods A 12-week randomized, double-blind, intervention on 44 patients with CVD. Patients were randomly allocated to receive either one LGG capsules $1.6 \times 10^9$ colony-forming unit (CFU) or the placebo capsules for 12 weeks. Gut microbiota profile and serum levels of interleukin-1β (IL-1β), Toll-like receptor 4(TLR4), interleukin-10(IL-10), lipopolysaccharide(LPS), and TMAO were assessed before and after the intervention.

Results A significant decrease in IL1-Beta concentration (-1.88 ± 2.25, vs. 0.56 ± 1.58 mmol/L, P=0.027), LPS levels (-5.20 ±2.70 vs. 2.96± 5.27 mg/L, P=0.016), and TMAO levels (-19.34±20.40 vs. -6.45±7.7 mmol/L, P=0.043) was observed after the probiotic supplementation compared with the placebo. L. rhamnosus statically increased in intervention group while Bacteroidetes non-significantly increased in both groups. Subjects who had ≥2.5 kg weight loss showed significantly improved in some variables, compared to patients with <2.5 kg weight reduction, regardless of the supplement they receive. Regression analysis revealed that baseline TMAO, changes of protein intakes and IL1-Beta predicted 58% of the final TMAO levels.

Conclusion: These data provide preliminary evidence that probiotic supplementation has beneficial effects on depressive symptoms, metabolic endotoxemia, TMAO, and gut microbiota profile in subjects with CVD.

Highlights
✔ Change of the intestinal microbiota (dysbiosis) has may be linked with risk of endotoxemia and CVD development.
✔ There is usually no acceptable treatment for this situation.
✔ The results our investigation display that probiotic led to decreased serum endotoxemia and TMAO
Introduction

Cardiovascular disease (CVD) is the main cause of death, morbidity, and disability in both western and developing country, with 1 in 3 people dying from CVD [1]. Despite the enormous growth in knowledge and advances in identifying traditional risk factors such as hypercholesterolemia, homocystinemia, hypertension, and hyperglycemia, there remain many questions about other cardiovascular risk factors yet [2, 3]. Currently the scholars have been paying attention to the role of gut microbiota alteration (dysbiosis) as one of the major etiological factors that are involved in the development of CVD [4].

Numerous data have supported the contribution of dysbiosis in development of CVD by some mechanisms including increased gut permeability and metabolic endotoxemia [5-7]. This can be explained by a microbiome-derived lipopolysaccharide (LPS), a major component of the external membrane in gram-negative bacteria. LPS can pass through the intestinal mucosa to enter the bloodstream, and may represent an important mediator of chronic inflammation [8]. Chronic inflammation following ME might be a possible mechanism for the association between dysbiosis and CVD which is triggered by dysbiosis [9]. LPS triggers toll-like receptors (TLRs) activation, and induces endothelial damage through increasing the expression of surface adhesion molecules such as cluster of differentiation 14 (CD14) on inflammatory cells, and stimulates the release of proinflammatory cytokines [10]. Endotoxin can also induce plaque formation and progression of atherosclerotic lesions, and release of other molecules from endothelial cells involved in proinflammatory process [11].

More recently, increased levels of Trimethylamine-N-oxide (TMAO), a gut bacterial metabolite, has been suggested as a new risk factor in CVD development [12]. Alteration in gut barrier function (dysbiosis) also leads to increased TMAO levels which is a “colorless amine oxide produced from betaine, choline, and carnitine via gut microbiota metabolism” [13]. It has been also revealed that TMAO levels substantially higher in individual with CVD compared to control subjects [12]. Additionally, TMAO levels were strongly associated with gut microbiota and newly it has been revealed that gut microbiota modulation by probiotics led to a decrease in TMAO levels [14].
Probiotics are well-defined as live microbe, which when administrated in adequate amounts, positively affect the health of the host by improving or restoring the gut flora. Specially, probiotics are exerting anti-obesity, lipid-lowering effects, anti-inflammatory and antioxidative activity as well [15]. Not only probiotics help keep balance in homeostasis of gut microbiota, they also have been considered as a possible treatment for CVD [16]. A small number of studies to date have explored the effects of probiotics on systemic levels of endotoxin in some clinical settings. Those that have observed the effect of probiotics administration on endotoxin levels and associated metabolic disorders have revealed conflicting results [17, 18]. Probiotics keep preserve integration of gut barrier function and decrease intestinal permeability, which consequently decline endotoxin and TMAO levels as well [19]. Weight loss diets and control of traditional risk factors are the treatment choices for CVD in overweight or obesity subjects [5]. Several guiding principle are recommend following a weight loss program to achieve 5–10% weight reduction [20]. Although the few animal studies appear to support the fact that dietary intervention leads to significant improvements in endotoxin levels [21], the effect of dietary intervention on endotoxin levels has not been studied in human researches yet.

To the best of our knowledge, there have been little or no controlled randomized trials testing the effect of probiotic supplementation along with calorie restriction on metabolic endotoxemia in coronary artery disease (CAD) subjects. Therefore, considering the anti-inflammatory, immune-regulatory, and gut microbiota balance effects of probiotics and the lack of clinical trials on therapeutic role of probiotics in patients with CAD, the current study evaluated the effects of probiotic supplementation along with calorie restriction on metabolic endotoxemia, TMAO and gut microbiota profile in CAD subjects.

Materials And Methods

Participants

We ran a double-blind randomized, placebo-controlled study to inspect the composition of the gut microbiota profiles, TMAO, endotoxemia, and inflammation levels in 44 CAD subjects. This trial was done at Shahid Madani Heart center serves as the heart center of Tabriz University of Medical Sciences, Iran, from July 2018 to February 2019. All patients admitted to this hospital with a diagnosis
of CAD were considered for participation in the study and screened by a cardiologist for eligibility. Patients were selected a few days after the PCI and/or conditions were stable. Inclusion criteria were as follows: having CAD; maintaining them during the course of the trial; normal diet; and Body Mass Index (BMI) = 25-35 kg/m². Patients were excluded if they refused to participate or had a low ejection fraction (< 35 %) or if they had a history of gastrointestinal disorders, if they had thyroid, renal, pancreatic, or liver diseases; if they were lactating, pregnant; if they are taking antibiotics, probiotics, prebiotics, inflammatory drugs one month before the intervention or during the intervention. A third party who was blind to the study gave the randomization sequence extracted from allocation software. The contributors, investigators and the medical providers were blinded after assignment to interventions. Informed consent was gotten from each participant. All 44 contributors completed the study; none dropped their participation (Fig. 1. CONSORT Flow diagram).

**Study design**

A blind trial at two levels (researcher, participants), placebo-controlled, randomized, clinical design was used to examine the effect of probiotic supplementation on endotoxemia in patients with CAD. Each qualified contributor was randomly allocated into probiotic or placebo group, according to 1:1 equal proportion rule. The order of random allocation was made by random sequence software. The inclusion criteria were matched for age, BMI, and sex. The random numbers were kept by a free person not complicated in the assessment of the patient, or in the data collection and analysis. In the current study, patients were randomly assigned into two groups to receive whichever probiotic supplements (n=22) or placebo (n=22) for 3 months. Both participants and researchers were unaware of the treatment allocation.

**Intervention**

Contributors in the probiotic group received one probiotic capsule daily containing a *Lactobacillus rhamnosus* $1.6 \times 10^9$ colony-forming unit (CFU) with their lunch. In the placebo (control) group (Tak Gen Zist Pharmaceutical Company, Tehran, Iran), the capsules contained maltodextrin. The physical properties of the placebo were identical in terms of shape, color, size, packaging, and smell but
contained no bacteria. Phone contacts were made to ensure adherence twice a month. Compliance to supplementation was established by requesting participants to return the medication containers. Also, for all the participants intended to a moderate calorie restriction dietary plan during 12 weeks’ intervention period. The program was planned to facilitate weight loss of 7-10% of weight, at a amount of 0.5-1 kg/wk throughout the intervention. Calorie intake was planned based on individual features of the subjects and with the aim of daily energy restriction (500 kcal fewer than the total energy requirements [TEE] estimated by Mifflin-St Jeor equation). The diets providing 55 ~ 60% of TEE from carbohydrate, 10 ~ 15% from protein, and 25 ~ 35% from fat.

**Dietary assessment**

Dietary intake was assessed using a dietary record at month 0, 1 and 3 of intervention. We used Nutritionist IV software adjusted for Iranian diets to acquire nutrient intakes of participants based on these average three-day food diaries.

**Physical Activity Assessment**

The physical activity assessment was gotten to monitor patients ‘usual physical activity levels throughout the study. The validated short-form International Physical Activity Questionnaire (IPAQ) was used to measure the participant’s physical activity. Based on previous studies, physical activities were classified as low, moderate, and high.

**Assessment of anthropometric and body composition**

Body weight was assessed via a scale with 250 gr accuracy (Seca, Hamburg, Germany) and patients were measured while wearing minimum dress and without shoes. Height without shoes was measured by a tape with 0.5-cm accuracy. BMI was computed by dividing weight (Kg) by height$^2$ (m). To avoid measurement bias, all date were taken by a trained dietitians.

Body fat mass, body fat percentage, and fat free mass (FFM) were assesed by the bioelectrical impedance (BIA) method via body composition analyzer (Takara BC- 418, Japan).

**Depression status**

The Beck Depression Inventory II (BDI-II) which is a self-report inventory with 21 items that measures the presence and severity of current (or past two weeks) depressive symptoms was used as measure
of depression. Items are valued on a 4-point scale ranging from 0 to 3 in terms of severity [22].

**Biochemical variables**

After an overnight fasting (12 hours), blood was collected and supplements provided to the participants. Blood serum was obtained from whole blood through centrifugation at 2500 rpm for 10 min. FPS and lipid profile were examined on the day of sampling, and residual serum was stored at -20 C until the analyses were done. Endotoxin, TMAO and inflammatory marker were measured using enzyme-linked immunosorbent assay [18] kits as follows: Interleukin 1 beta (IL-1β) (intra-assay variation, 5.8%, normal range 0.02-6 pg/mL, inter-assay variation, 9.06%, sensitivity = 0.01 pg/mL), Toll Like Receptor 4(TLR-4) (normal range 0.05-15 ng/mL, intra-assay variation, 4.58%, inter-assay variation, 7.8%, sensitivity = 0.027 ng/mL), IL-10 (normal range 0.2 100 ng/dl, intra-assay variation, 10%), TMAO (sensitivity 0.23 pg/ml, inter-assay CV = 5.5%, detectable range 5.00 – 150 pg/ml), LPS (inter-assay CV = 10.0%, detectable range 12.00 – 1000 ng/ml).

Serum high sensitivity C-reactive protein (hs-CRP) levels were evaluated by immunoturbidimetry. Serum concentration of malondialdehyde (MDA) was based on the reaction of MDA with thiobarbituric acid and the serum total antioxidant capacity [23] was determined by commercially available kits (Glory Science Co). Lipid profile containing total cholesterol, triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) levels were assessed by enzymatic kits (Pars Azmun, Iran). Friedewald formula was done in order to determine low-density lipoprotein cholesterol (LDL-C) levels. Fasting blood sugar (FBS) level was measured via the glucose oxidase technique through commercial kit (Pars Azmun, Iran).

**Blood pressure**

Diastolic blood pressure (DBP) and systolic blood pressure (SBP) were measured in seated subjects, after 5 minutes of rest, by an automatic oscillometric device (Omron Healthcare Co, Ltd) before and after treatment.

**DNA extraction and real-time PCR analyze for evaluation of gut microbiota**

Morning fecal samples were assembled from subjects before and after intervention via a sterile container, which was then transported to the laboratory in a frozen condition and stored at -80°C till
analysis. We assessed the quantity of Firmicutes, Bacteroidetes and *L. rhamnosus* that were included in the probiotic mixture. For DNA extraction of gut microbiota, 1 g of each stool samples were dissolved in 9 mL of phosphate-buffered saline and then centrifuged for 2 minutes, and at that point 200 mL of each sample was taken for DNA extraction, using a QIAamp DNA stool mini-kit following the manufacturer’s procedures (Qiagen, Hilden, Germany). The extracted total DNA was well-kept at −20 °C. Real-time PCR (SYBR green method) was performed for each samples using qRT-PCR (the Rotor gene 3000, Corbett Life Science, Mortlake, Australia), which each RT-PCR reaction contained 10 mM of each primer of Firmicutes, Bacteroidetes and *L. rhamnosus*, 5 µl SYBR Green Master mix (Fermentase, Waltham, MA, USA) and 2 µl DNA template in a 10 µl reaction volume. The primers were synthesized by Pishgam (Pishgam, Biotech, Tehran, Iran), which were shown in Table 1. The amplification conditions were carried out as follows: 2 min at 94 °C as the first denaturation; 35 cycles of 30 s at 94 °C, 30 s at 61 °C 59 °C 57 °C for Firmicutes, Bacteroidetes and *L. rhamnosus*, respectively and 30 s at 72 °C and 10 min at 72 °C as the final extension.

**Statistical analysis**

The data were analyzed via SPSS software (version 21; SPSS Inc., Chicago, IL) and the outcomes were stated as mean ± SD. To determine the normal distribution of variables, we used skewness and kurtosis test. Within-group comparisons (end-point vs. baseline) was applied by paired samples t-test. To adjust for several confounders, we done analysis of covariance (ANCOVA) in which the confounding effect of these variables were taken into account which was used to determine the statistically significant pairwise differences. The analyses were conducted using an intention-to-treat approach [24]. Circulating endotoxin and TMAO level was intended as a primary outcome, while anthropometrics, FBS, lipid profile, inflammatory markers, and gut microbiota profile were defined as secondary outcomes. To investigate the predictors of change in TMAO level, hierarchical step-wise regression analysis was performed; TMAO score was entered as the dependent variable, and the predictors investigated were: baseline TMAO levels, Protein intakes *L. Rhamnosus*, Bacteroid, Firmicutes, IL1-B, IL-10, LPS TLR-4, hs-CRP, and difference between baseline and final values of weight. For evaluating clinical importance of probiotic administration, number needed to treat (NNT)
was calculated using the standard method (inverse of the risk difference), and based on ≥3 points reduction in BDI score. For all statistical tests, a P value less than 0.05 was interpret as statistically significant.

Results

Figure 1 show the study flowchart; ninety patients stated interest in the study and 60 who met the eligibility criteria were enrolled. Of these 60 patients, 44 (73%) met the inclusion and exclusion eligibility criteria. A total of 22 patients were randomly assigned to the placebo group and 22 to the probiotics group. Hence, a total of 44 patients completed the study, 1 patient in the placebo and 2 patients of the probiotic group reported symptoms including gastrointestinal problems and upset stomach. These findings indicate that most patients tolerated probiotics very well.

Baseline medical and demographic data are presented in Table 2. There were no significant differences between the groups regarding weight, BMI, physical activity levels, and family history of CAD. BDI-II depression scores ranged from 3 to 63, and the mean baseline BDI-II score did not differ between the probiotics group (20.12; 95% confidence interval [CI], 16.27-23.72) and placebo group (17.70; 95% CI, 15.11-20.10) at baseline ($P = 0.266$).

Data on dietary intakes (macronutrient distribution {as percentage of calories} changes and dietary fiber) of the patients are summarized in Table 3. Intake of calories and macronutrients significantly decreased in both groups. We found significant change in group mean energy intake during the intervention (decrease of -284.40 and -384.71 Kcal in probiotics and placebo group, respectively, $P=0.225$). Greater decrease dietary intake was observed in the prebiotic group compared to placebo ($P>0.05$). Although did not reach statistical significance compared to placebo at endpoint of the study.

Percent changes (PCs) for anthropometric measures, and blood pressure are demonstrated in Figure 2. As presented, weight, BMI, waist circumferences, body compositions DBP, and SBP declined in both groups; but, the between-group differences were non- statically significant after intervention while adjusted for the potential confounding factors (baseline and calorie intake).

Median PCs for lipid profile, and FBS are depicted in Figure 3. Changes for some variables (TC, LDL,
and BDI) reached statistical significance.

PCs for TAC, MDA, and hs-CRP are demonstrated in Figure 4. A significant difference in serum TAC concentrations (236.7±88.4 vs. 168.54±64.7 mmol/L, P=0.009), hs-CRP levels (1.26 ±0.70 vs. 1.93+1.27 mg/L, P=0.040) and MDA levels (110.7 ±63.73 vs. 156+67.6 nmol/mL, P=0.033) were detected after the probiotic intervention compared with the placebo.

Also, twelve patients in probiotics group experienced ≥3 point’s improvement in BDI score; thus, the calculated NNT for 12-weeks probiotics supplementation to reach a minimum decrease of three points on BDI score was 6 persons (95% CI: 2 to 8) (Data not shown).

**Table 4** provides a summary of pre- and post-intervention metabolic endotoxmia and TMAO levels in both groups. Neither the between-group differences, nor the within-group variations reached statistical significance for TLR4. A significant decrease in IL1-Beta concentration (-1.88 ± 2.25, vs. 0.56 ± 1.58 mmol/L, P=0.027), LPS levels (-5.20 ±2.70 vs. 2.96+ 5.27 mg/L, P=0.016), and TMAO levels (-19.34±20.40 vs. -6.45±7.7 mmol/L, P=0.043) were detected following the probiotic supplementation compared with the placebo. Accordingly, probiotics administration resulted in a significant decrease in biomarker levels of inflammation, metabolic endotoxmia and TMAO compared with the placebo.

A significant increase in *L. rhamnosus* DNA expression (245/01 ±77.40 vs. 15±64.7 CFU, P=0.001), Bacteroid DNA expression (0.15 ±0.03 vs. 0.12±0.03 CFU, P=0.443), and decrease in Firmicutes levels (-0.35 ±0.09 vs. -0.13 ±0.10 CFU, P=0.392) were detected following the supplementation with probiotic compared with the placebo. Thus, taking probiotic resulted in a decrease in dysbiotic bacterial genera, and increase in favorable levels of gut microbiota compared with the placebo (**Table.5**). Improvements in gut microbiota were also stronger in patients receiving probiotic plus calorie restriction than in those receiving placebo.

Results stratified by weight reduction of at minimum 2.5 kg was presented in **Table 6**. Overall, 28% of the participants who finished the trial had at least 2.5 kg weight loss (8 patients in the probiotic group and 4 patients in the placebo group). Nevertheless, besides of what supplement they receive, subjects who reached weight loss, had great more decrease in some biomarkers in comparison with
who lost <2.5 kg.

**Table 7** presents three models for forecasting TMAO levels among the total study sample. Model 3, being the most suitable one, can predict approximately 58% of the changes observed in the dependent variable; it includes baseline TMAO, as well as changes of proteins and IL1-Beta, as independent variables. According to this model, for every 1-gram raise in proteins, and every 1-unit increase in IL1-Beta circulating levels, TMAO is expected to increase by 0.38 (P=0.050) and 3.33 (P=0.048) units, respectively.

**Discussion**
As far as we know, this is the first study to provide insight into the anti-inflammatory and anti-endotoxemia effects of probiotics in CAD patients. The results of the current study indicated, for the first time, that weight loss diet along with probiotics supplementation lead to improved endotoxemia, as described by reduction of the LPS and TMAO levels. Probiotic administration was also improved gut microbiota profile. In addition, inflammatory and oxidative stress markers were influenced favorably by probiotic supplements, among patients with CAD under a calorie-restricted diet. We also came to the conclusion that those who were obedient with the weight loss diet and lost a minimum of 2.5 kg by the end of intervention had significantly improved metabolic profile compared to those with slight compliance with the diet.

In the current study, we ran the weight loss program plus probiotic supplementation. Although anthropometric and body composition indices reduction in the probiotic group was more advantage than the placebo group, this issue did not range to statically significant levels. Similar consequences have been stated with probiotics supplementation in former studies [25]. In the current study, we did observe statistically major changes in nutrient intakes for the period of the intervention, there was a downward trend in the total energy intake in all subjects, because we ran weight loss diet for both groups. Though weight loss was similar between the two groups, our results presented here suggest that weight loss diet offers a favorable effect on metabolic disorders, but add-on the diet plus probiotics may promote cardiovascular risk factors more (Table 6).

The other result of probiotic supplementation in CAD patients was an improvement in inflammatory
markers. To the extent of our knowledge, no study has assessed the effect of probiotics supplementation on endotoxemia in patients with CAD. Former studies have stated microbial dysbiosis in CVD subjects [8]. Probiotic supplementation by modulating gut microbiota enhances the immune system function and decreases inflammation and endotoxemia [9, 17]. In our study, we establish a statistically significant reduction in LPS levels in probiotic group compared with placebo, but not in TLR4, suggestive of probable anti-inflammatory and anti-endotoxemia properties of probiotics. Activation by LPS resulted in an increase of plasma levels of cytokines like interleukin (IL-) 1 Beta which observed in CAD patients [8]. The outcomes of our study show that probiotic supplementation led to decreased IL-Beta and hs-CRP levels. The anti-inflammatory effects of probiotics may involve the production of the of short chain fatty acids (SCFA) in the gut microbiota and the decreased expression of inflammatory cytokines [26]. This finding is consistent with the outcomes reported by Zarrati et al [27], who established that Lactobacillus acidophilus administration as a probiotic, for 2 month resulted in a significant decrease in inflammatory markers among obese subjects. In addition, a significant decrease in hs-CRP levels was detected after the intake of probiotic yogurt for 9 weeks among pregnant women [28]. Others have failed to find a significant effect of two-month probiotic supplementation on CRP levels in polycystic ovary syndrome (PCOS) subjects [29]. The different results might be clarified by diverse dosages of probiotics supplementations and different clinical setting of individuals who took part in those studies. Microbial translocation has been proposed to be a driver of inflammation and immune activation in CAD patients [6, 30]. Whereas the contribution of pro-inflammatory cytokines to progression of CVD is well-recognized [31], little is identified about the effect of microbial translocation (increases LPS) on higher cytokine secretion [32]. To the best of our knowledge, no clinical trial had appraised the effects of probiotics on TLR-4 in meta-inflammation. TLR4 has been suggested to act as a molecular link among LPS, inflammation and the innate immune system [11]. It seems that probiotic supplements reduce inflammation possibly via competitive mechanism which prevents binding LPS to theTLR4/CD14 complex [9, 19]. In addition, previous studies have indicated that calorie restriction can downregulate LPS-producing enzymes of the commensal bacteria, leading to decreased endotoxemia and
inflammation [33]. There is one single study which indicates that being on a very-low calorie diet (800 Cal/day) for one month decreases LPS binding protein and zonulin in obese women [34]. Apart from that, there are few clinical trials available about the effects of calorie restriction (25–30% below TEE) on serum LPS and TMAO. Therefore, we could not compare our results for LPS and TMAO, to the previous studies about the effect of calorie restrictions on these factors.

Previous epidemiological studies have proposed direct associations of increased TMAO levels with greater risk for myocardial infarction, stroke and death [9]. Gut microbiota profile plays a pivotal role in the formation of TMAO from dietary carnitine and choline [6, 12]. Animal studies also have shown the favorable effects of the probiotic administration on TMAO levels and metabolic endotoxemia [14]. Our results did confirm these findings in patients with CAD who took LGG supplementation. In contrast with our results, Tripolt and colleagues reported that supplementation with probiotics containing Lactobacillus Casei Shirota had no effect on TMAO levels in human studies [35]. Apart from this study, there was no clinical trial which has been done to evaluate the effects of probiotic supplementation on TMAO levels.

It has been revealed that TMAO levels are related with gut microbiota at phylum as well as family levels. On the contrary, we did not observe statistically significant correlation between TMAO levels with gut microbiota profiles in the present study (the data are shown). The main explanation is that we did not measure the whole gut microbiota and therefore we failed to observe this association.

As mentioned above, dysbiosis leads to endotoxemia and chronic inflammation, as well as increased TMAO levels. These two factors have been proposed to make subjects vulnerable to CVDs. Probiotics might reverse these effects by helping to enhance or restore health gut microbiota composition. Our results show that probiotic supplementation significantly increased the stool concentration of Lactobacillus and, and reduced in stool concentrations of Firmicutes and increased Bacteriodes in the probiotic group. These results are in agreement with those reported by Cui et al.[36], where the probiotic supplementation with Bifidobacterium spp in IBD patients for 2 months, meaningfully increased the stool concentration of Bifidobacterium spp and Lactobacillus spp, compared to placebo. However, in the study of Kato et al.[37], consumption of Bifidobacterium-fermented milk for 3 months
did not alter the level of stool Bacteriodes. This variety in findings may be caused by the different gut microbiota status, the dose and type of probiotics, and the clinical state of the patients.

Another important aspect of our findings is that increases Bacteroid and decrease in Firmicutes levels occur after only the weight loss program. There was a little clinical trial which has been conducted to assess the effects of weight loss programs on gut microbiota profiles. Some Previous studies show that changes in the proportion Firmicutes-to-Bacteroidetes ratio were found after Roux-en-Y gastric bypass (RYGB) surgery [38, 39], whereas other in studies demonstrated that the total numbers of bacteria decreased after calorie restriction [23, 40]. These inconsistencies may possibly have attributed to considerable variances in levels and duration of calorie restriction in different clinical setting. Taken together, the results presented here are a significant basis for further clinical interventional trials.

Patients with CAD are predisposed to experience several challenges including lipid metabolism disorders and increased risk of depression [41, 42]. Another finding of the current trial is that taking probiotic supplements in CAD patients for 12 weeks resulted in significant reduction in depression level as indexed by The BDI-II questionnaire and improved lipid profile compared with the placebo. NNT also was high (NNT = 6) for the probiotic supplementation in our study. Several studies have evaluated those effects of probiotic supplementation [43, 44]. Probiotic supplementation has been reported to improve depression symptom, through increasing serotonin metabolite levels in the brain, and decreasing endotoxemia [45, 46]. All in all, we might propose that weight loss programs plus probiotic supplementation could be another choice of priority for depression symptom and metabolic disorders which are common in CAD patients.

Our study has some of the limitations, including not assessing the whole of gut microbiome instead of the level of Firmicutes and Bacteriodes, as well as the duration and doses of the supplementations. Nevertheless, given the gradually common use of Firmicutes/Bacteriodes ratio, the analysis of these bacterial contents may well be used in the future as indicators of a dysbiotic microbiome that contributes to low grade inflammation and CVD progression. Apart from that, this supplementation is worthy of additional survey.
Conclusion
We perceived an alter the composition of gut microbiota subsequent probiotic supplementation (L. rhamnose), resulting in decreases of endotoxmia and inflammation. The use of probiotics may be a promising therapeutic strategy for CAD patients. In addition, our results propose that calorie restriction plus probiotic supplementations might lead to improved metabolic endotoxemia and TMAO levels, better than supplementation alone.

Abbreviations
MI: Myocardial infarction; LVEF: Left ventricle Ejection fraction; ELISA: enzyme-linked immunosorbent assay, HF: heart failure, BMI: Body Mass Index BP: Blood Pressure DBP: Diastolic Blood Pressure, FBS: Fasting Blood Sugar, HDL: High-Density Lipoprotein, LDL: Low-Density Lipoprotein, SBP: Systolic Blood Pressure, TC: Total Cholesterol, TG: Triglyceride

Declarations

Ethics declarations

Conflict of Interest
The authors declare that there is no conflict of interest.

Ethical Approval
Our study was in agreement with the Helsinki Declaration of the World Medical Association (2000) and was accepted by our local ethics committee of Tabriz University of Medical sciences as a proposal for PhD grade in Nutritional Sciences (IR.TBZMED.REC.1397.184) and also was listed in the Iranian Registry of Clinical Trials (IRCT) (IRCT20121028011288N15).

Consent for publication
All authors support the submission to this journal.

Informed Consent
Informed consent was obtained from all individual participants included in the study using opt-out procedure.

Funding/Support
This research was partially supported by Tabriz University of Medical sciences ((grant number 184).

Acknowledgements
We express thanks all members of current study group who providing understanding and expertise that greatly assisted the research.

**Author contribution**

Jalal moludi designed the study. Hossein Samadi Kafil and Pourya Gholizadeh completed the entire clinical studies. Jalal moludi collected and analyzed the data. Mohammad Alizadeh prepared the manuscript. Jalal moludi conducted statistical analysis. All of authors edited the manuscript.

**Availability of data and materials**

All data generated and analyzed during this study are included in the manuscript.

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Tables

| Table 3. Changes in calorie, percent from macronutrients, and dietary fiber throughout the study |
|---------------------------------|----------------|----------------|---|
| Variable                        | Probiotic group (n=22) | Placebo group (n=22) | P-value |
| **Energy (Kcal/d)**             |                |                | 153.4 (-116.1, 53.27) |
| Baseline                        | 2214.14 (428.6) | 2060.7 (454.38) | 0.257 |
| End                             | 1829.34 (174.6) | 1776.1 (184.3) | 0.225 |
| MD (95% CI), P*                  | -384.71 (-567.9 to -201.6), <0.001 | -284.40 (-447.40 to -121.9), <0.001 | |
| **Carbohydrates (%)**           |                |                | 0.80 (-7.2, -0.92) |
| Baseline                        | 53.33 (13.43)  | 54.1 (11.51)   | 0.987 |
| End                             | 54.72 (10.22)  | 53.88 (11.34)  | 0.807 |
| MD (95% CI), P*                  | 1.39 (-7.12, 7.66), 0.667 | -0.25 (-6.34, 5.12), 0.672 | |
| **Protein (%)**                 |                |                | 0.44 (-2.2, 0.62) |
| Baseline                        | 13.55 (3.9)    | 13.99 (3.76)   | 0.087 |
| End                             | 15.08 (3.68)   | 15.7 (2.76)    | 0.672 |
| MD (95% CI), P*                  | 1.53 (-3.44, 2.21), 0.172 | 1.71 (-2.21, 0.72), 0.634 | |
| **Fat (%)**                     |                |                | 1.01 (-6.2, -2.01) |
| Baseline                        | 34.12 (9.87)   | 32.1 (7.72)    | 0.751 |
| End                             | 31.20 (10.62)  | 31.12 (10.85)  | 0.756 |
| MD (95% CI), P*                  | -4.08 (-7.21, 3.33), 0.662 | -2.00 (-4.12, 5.31), 0.656 | |
| **Dietary fiber (g)**           |                |                | 3.37 (-0.06, 0.34, 0.090) |
| Baseline                        | 16.40 (4.25)   | 15.72 (3.17)   | 0.090 |
| End                             | 19.77 (8.05)   | 17.72 (6.51)   | 0.147 |

Mean (SD) and Mean difference (95% CI) are presented for data.
* P based on Paired samples t-test
** P based on Independent samples t-test
*** P based on ANCOVA adjusted for baseline values
Table 1. Primers and probes used for detection of each bacterium

| Primers               | Sequence                  |
|-----------------------|---------------------------|
| **Firmicutes**        |                           |
| Forward               | TCCTACGGGAGGCAGTAG         |
| Reverse               | TACGTATTACCGCGGCTGCTG     |
| **Bacteroidetes**     |                           |
| Forward               | GAGAGGAATCCCCCCAC         |
| Reverse               | GCTACTTGGCTGGTTCAG        |
| **L. rhamnosus**      |                           |
| Forward               | TGATTATTGAAGGTGCTTGCAT    |
| Reverse               | TTAGCCATCTTTTACGCAAG      |

|                          | Probiotic group (n=22) | Placebo group (n=22) | P-value   |
|--------------------------|------------------------|----------------------|-----------|
| at study                 | 56.7 (9.1)             | 57.1 (7.8)           | 0.876d    |
| after                    | 75.6 (12.3)            | 79.2 (12.1)          | 0.390d    |
| ' at study               | 72.35 (12.4)           | 77.54 (11.2)         | 0.156     |
| ' at study               | 27.33 (3.8)            | 27.6 (2.6)           | 0.832d    |
| 20 (90)                  |                        | 21 (95)              | 0.0950    |
| 13 (61.9)                | 12 (63.2)              |                      | 0.935     |
| 5 (22)                   | 4 (18)                 |                      | 0.431c    |
| 773.7 (198.3)            | 760.6 (201.21)         |                      | 0.830     |
| 37.7 (7.1)               | 38.6 (8.2)             |                      | 0.527     |
| 20.10 (8.1)              | 17.61 (4.7)            |                      | 0.266     |

*ary artery diseases, METs: Metabolic equivalents (MET-minutes/week); MD: Mean/Median of difference
*e expressed as mean (SD)
*re expressed as frequency (%)
e test
tent samples t-test
Table 4. Effect of probiotics supplementation on metabolic endotoxemia and TMAO levels

| Variable       | Probiotic group (n=22) | Placebo group (n=22) | P-value          |
|----------------|------------------------|----------------------|-----------------|
|                | Baseline               | End                  | Baseline        | End                  |                      |
| **IL-10 (ng/dl)** | 5.11 ± 4.6             | 4.76 ± 3.6           | 0.11 (-2.4)     |                      |                      |
|                | 5.25 ± 2.91            | 5.78 ± 2.29          | 0.56 (-2.6)     |                      |                      |
| MD (95% CI), P* | 0.60 (-1.4 , 2.6)      | 0.56 (-1.31 , 3.59)  | 0.399           |                      |                      |
| **IL1-Beta (pg/mL)** | 5.62 ± 3.72            | 5.31 ± 2.09          | -0.31 (-1.4)    |                      |                      |
|                | 3.75 ± 2.1             | 5.81 ± 3.6           | 2.05 (-3.8)     |                      |                      |
| MD (95% CI), P* | -1.88 (-3.25 , -0.48)  | 0.56 (-1.58 , 2.56)  | 0.546           |                      |                      |
| **LPS (ng/ml)** | 21.92 (11.64)          | 26.18 (16.85)        | 4.25 (-13)      |                      |                      |
|                | 16.04 (6.8)            | 23.22 (12.22)        | 7.27 (-13.4)    |                      |                      |
| MD (95% CI), P* | -5.92 (-10.74 , -1.09) | -2.96 (-11.6 , 5.7)  | 0.165           |                      |                      |
| **TLR4 (ng/ml)** | 11.59 (7.22)           | 10.58 (8.30)         | -0.70 (-2.1)    |                      |                      |
|                | 9.8 (7.24)             | 11.85 (5.6)          | 2.2 (-5.3)      |                      |                      |
| MD (95% CI), P* | -1.72 (-6.21 , 3.10)   | 1.27 (-1.7 , 4.7)    | 0.581           |                      |                      |
| **TMAO (pg/ml)** | 36.55 (38) 65          | 41.94 (44) 18        | 4.53 (-30.6)    |                      |                      |
|                | 17.67 (6) 30           | 34.61 (36) 72        | -17.4 (-34.2)   |                      |                      |
| MD (95% CI), P* | -019.0 (55.3 - 63.5)   | -0.165 (8.3 - 7.15)  | 45.6            |                      |                      |

LPS: Lipopolysaccharides TMAO: Trimethylamine-N-oxide, Mean (SD) and Mean difference (95% CI) are presented for data.
* P based on Paired samples t-test
** P based on Independent samples t-test
*** P based on ANCOVA adjusted for baseline values
### Table 5. Effect of probiotics supplementation on gut microbiota profile including stool concentrations of lactobacillus rhamnosus and Bacteriodes

| Variable          | Probiotic group (n=22)          | Placebo group (n=22)          | MD (95% CI), P-value |
|-------------------|--------------------------------|--------------------------------|----------------------|
| **L. rhamnosus**  |                                |                                |                      |
| Baseline          | 2.93 ± 1.74                   | 2.66 ± 1.70                   | 0.001                |
| End               | 248.30 ± 78.30                | 11.23 ± 38.29                 |                      |
| MD (95% CI), P*   | 245/01 (211/0, 278/1)         | 15/58 (-8/-70, 25/8)          | 0.001                |
| **Firmicutes**    |                                |                                |                      |
| Baseline          | 0.62 ± 0.78                   | 0.46 ± 0.44                   |                      |
| End               | 0.26 ± 0.31                   | 0.31 ± 0.32                   |                      |
| MD (95% CI), P*   | -0/35 (-0/-10, -0/-14)        | -0/13 (-0/-30, 0/11)          | 0.008                |
| **Bacteroid**     |                                |                                |                      |
| Baseline          | 0.16 ± 0.21                   | 0.15 ± 0.16                   |                      |
| End               | 0.28 ± 0.24                   | 0.33 ± 0.25                   |                      |
| MD (95% CI), P*   | 0/08 (0/05, 0/31)             | 0/11 (0/02, 0/20)             | 0.011                |

CFU: Colony-forming unit,
Mean (SD) and Mean difference (95% CI) are presented for data.
* P based on Paired samples t-test
** P based on Independent samples t-test
*** P based on ANCOVA adjusted for baseline values

### Changes in biomarkers and variables among the patients who completed the study, stratified by a decrease of 2.5 kg weight

| Variable          | weight loss≤2.5 (n=12) | weight loss≥2.5 (n=32) | MD (95% CI), P-value |
|-------------------|------------------------|------------------------|----------------------|
| Total cholesterol | 140.83 ± 43.16         | 159.66 ± 47.11         | -18.23 (-13.14 to 50.21), 0.241 |
| weight loss (mg/dl) | 134.34 ± 37.16         | 160.83 ± 59.25         | -25.52 (-11.1 to 62.33), 0.174 |
| 72.36 ± 44.16     | 87.44 ± 44.39          | -15.11 (-15.1 to 45.2), 0.326 |
| 42.00 ± 6.27      | 44.31 ± 7.59           | -2.31 (-2.2 to 7.1), 0.353 |
| 113.33 ± 13.18    | 122.47 ± 12.16         | -9.13 (-0.26 to 18.31), 0.056 |
| 78.83 ± 7.16      | 77.10 ± 8.10           | -0.99 (-8.66 to 6.51), 0.794 |
| 13.50 ± 3.80      | 16.71 ± 4.33           | -3.21 (1.47 to 6.2), 0.035 |
| 41.75 ± 6.13      | 39.28 ± 7.23           | -2.46 (-7.7 to 2.31), 0.301 |
For abbreviations see table 1
Values are expressed as mean (SD)
a- Independent samples t-test

|   |   |   |
|---|---|---|
| hs-CRP (mg/dl) | 1.14 ±0.45 | 1.79±  1.25 | -0.65 (0.16 to 0.24), **0.011** |
| TMAO (mmHg) | 125.16 ±44.16 | 137.00± 72.39 | -11.83 (-35.1 to 59.71), 0.616 |
| IL1-Beta (mmHg) | 219.66 ±64.83 | 193.81 ±89.50 | -25.83 (-83.1 to 31.32), 0.367 |
| IL-10 (mmHg) | 23.90 ±10.18 | 26.81 ±33.29 | -2.91 (-16.1 to 22.2), 0.769 |
| L. Rhamnosus (CFU) | 4.17 ±1.93 | 5.10 ±3.43 | -0.84 (-1.21 to 2.94), 0.429 |
| Bacteroid (CFU) | 4.05 ±2.11 | 6.09 ±3.99 | -2.04 (-0.44 to 4.50), 0.101 |
| Firmicutes (CFU) | 10.47 ±4.30 | 16.25 ±9.75 | -5.74 (-0.22 to 11.54), 0.059 |
| U | 19.50± 11.50 | 20.18± 10.42 | -0.77 (-6.1 to 8.81), 0.833 |
| U | 4.17 ±1.07 | 4.50±  1.23 | -0.32 (-1.09 to 0.44), 0.404 |
| U | 19.41±11.42 | 19.68± 9.75 | -0.27 (-6.7 to 7.2), 0.938 |
| U | 10.11±6.81 | 11.11± 6.39 | -1.00 (-3.1 to 5.40), 0.650 |
| U | 181.66±151.83 | 110.17±123.50 | -71.46 (-161.1 to 18.2), 0.116 |
| U | 0.37±0.27 | 0.28±0.22 | -0.08 (-0.24 to 0.07), 0.309 |
| U | 0.23±0.30 | 0.32±0.31 | -0.08 (-0.13 to 0.30), 0.777 |

*ons see table 1
pressed as mean (SD)
t samples t-test
Table 7. Hierarchical Multiple regression analysis for predicting final TMAO levels

| Independent Variables | Model 1 |   |   |   | Model 2 |   |   |   | Model 3 |   |   |   |
|-----------------------|---------|---|---|---|---------|---|---|---|---------|---|---|---|
|                       | B       | SE| Beta| P | B       | SE| Beta| P | B       | SE| Beta| P |
| Baseline TMAO         | 0.48    | 0.07| 0.69|0.001| 0.49    | 0.70| 0.71|0.001| 0.52    | 0.07| 0.75|0.001|
| Weight change*        | 0.78    | 0.68| 0.12|0.295| 0.75    | 0.67| 0.11|0.271| 1.06    | 0.68| 0.16|0.130|
| Protein intakes       | 0.30    | 0.20| 0.16|0.138| 0.38    | 0.18| 0.20|0.050| 0.43    | 0.22| 0.23|0.068|
| L. Rhamnosus          | -0.03   | 0.20| -0.17|0.113| -0.04   | 0.02| -0.21|0.096|         |     |     |     |
| Bacteroid             | 14.20   | 19.39| 0.11|0.464| 20.9    | 19.66| 0.17|0.294|         |     |     |     |
| Firmicutes            | -16.24  | 17.05| -0.15|0.347| 11.7    | 17.38| 0.11|0.504|         |     |     |     |
| IL1-B                 |         |     |     |     | 3.33    | 1.62| 0.33|0.048|         |     |     |     |
| IL-10                 |         |     |     |     | 1.98    | 1.24| 0.24|0.134|         |     |     |     |
| LPS                   |         |     |     |     | 0.12    | 0.34| 0.04|0.730|         |     |     |     |
| TLR-4                 |         |     |     |     | 0.08    | 0.54| 0.20|0.872|         |     |     |     |
| hs-CRP                |         |     |     |     | 3.12    | 3.01| 0.11|0.308|         |     |     |     |

Adjusted R² %: 53, 57, 58

*changes from baseline of the study; TMAO: Lipopolysaccharide; IL-10: Interleukin-10; Dependent variable was TMAO levels at endpoint of the study

Figures
Figure 1

flowchart of study
Figure 2

Effect of probiotic on anthropometric indices and body composition

Figure 3

Effect of probiotic on lipid profiles
effect of probiotic on oxidative stress AND inflammation biomarkers