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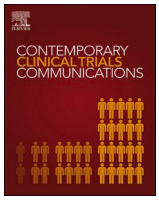

Impact of probiotics on gut microbiota composition and clinical symptoms of coeliac disease patients following gluten-free diet

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ABSTRACT

Coeliac disease (CD) is associated with alterations in gut microbiota composition. This study evaluated the effects of probiotics on gut microbiota composition and clinical symptoms of treated CD patients. In this double-blind, placebo-controlled trial study, 31 CD patients that were randomly classified as probiotics (n = 15) and placebo (n = 16) groups received 10^9 colony-forming units/capsule for 12 weeks. Fecal samples were collected before and after probiotics, or placebo administration and the changes in intestinal microbiota were assessed by quantitative real-time PCR. Probiotic administration improved the patients' clinical symptoms when compared to the placebo group. Fatigue score was significantly reduced by the intake of probiotic supplements (P = 0.02). Except for *Staphylococcus* spp., the relative abundances of *Bacteroidetes*, *Lactobacillus* spp., *Bifidobacterium* spp., *Clostridium* cluster I, *Enterobacteriaceae*, and *Firmicutes* were higher in probiotics group. Accordingly, a 12-week multi-strain probiotic treatment regimen may modify the composition of intestinal microbiota and improve GI symptoms in CD patients.

1. Introduction

Coeliac disease (CD) is an immune-mediated systemic disorder of the small intestine triggered by exposure to dietary gluten in genetically predisposed individuals [1]. Gluten consumption induces an inflammatory cascade in the small intestinal mucosa that leads to villous atrophy, crypt hyperplasia, increased numbers of lymphocytes in the lamina propria, and consequently poor absorption of nutrients [2,3]. According to initial prevalence studies in the general population from European countries, the prevalence of CD is approximately 1% of the European population. Gut microbiota, genetic predisposition and environmental factors such as dietary gluten are important factors involved in CD [4,5]. There are few studies about the gut microbiome and its role in CD, however, it has been shown that the gut microbiota composition of CD patients is different from healthy controls [1,6,7]. It is not clear if dysbiosis of microbiota composition plays a role in the pathogenesis of the disease, or whether it is just a consequence of inflammation in CD [1]. The majority of studies have reported that dysbiosis in fecal and duodenal specimens of CD patients is characterized by higher numbers of Gram-negative bacteria (*Bacteroides* and *Enterobacteriaceae*) and decreased the number of beneficial Gram-positive bacteria (*Bifidobacterium* spp.) in comparison to healthy individuals [8,9].

Currently, a gluten-free diet (GFD) is the only available and proven treatment for CD [10,11]. Compliance with a GFD can be a challenge for many CD patients and several factors including social and practical problems such as lack of knowledge about the diet, non-availability of gluten-free foods, social pressure, temptation and not liking the taste of foods made of alternative food grains have associated with noncompliance to gluten-free dietary regimen [12,13].

Given the challenges of adhering to a gluten-free diet (GFD) for many

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individuals, it is crucial to explore complementary approaches to enhance the treatment of CD [14,15]. Also, despite strict adherence to a GFD, many CD patients do not experience symptomatic improvement [16]. According to previous reports, CD patients with persistent symptoms on GFD may experience an altered intestinal microbiota resembling those of IBS subjects [17,18]. Recently, probiotics containing diverse bacterial strains is proposed as adjuvant therapy in conjunction with GFD [16]. Probiotics usage may be a safe supplement to reduce the severity of symptoms. Some studies showed that probiotics can modulate both the innate and adaptive immune system, and mitigate gliadin-induced inflammation [19–21]. Also, it has been suggested that probiotics usage restores a normal proportion of beneficial bacteria and helps reduce imbalances in the intestinal microbiota of the gastrointestinal (GI) tract [22]. Moreover, *Lactobacillus fermentum* or *Bifidobacterium lactis* are shown to promote gluten-degrading properties *in vitro* [23]. Despite *in vitro* studies, the effects of probiotics on CD have been poorly explored *in vivo* [24]. Regarding intestinal dysbiosis in CD and the role of gut microbiota in regulating the immune system, it is suggested that probiotics may be able to modify the intestinal microbiota of CD patients and also relieve the patients' GI symptoms. As CD is reported to be accompanied by an imbalance in Gram-positive to Gram-negative bacteria ratio [8,9], in the present study we quantified the relative abundances of the most abundant phyla, such as *Firmicutes* (including Gram-positive bacteria like *Staphylococcus*, *Clostridium*, *Lactobacillus*), *Bacteroidetes* (Gram-negative) and *Bifidobacterium* (as main subgroup of *Actinobacteria* (Gram-positive)) in the fecal samples of study population. The family *Enterobacteriaceae* was also selected as Gram-negative bacteria that belongs to the potentially harmful *Proteobacteria* phylum, which are associated with inflammatory responses in the small intestine [25]. We examined the effects of probiotics supplementation on alterations of a selection of intestinal microbiota taxa and clinical symptoms among treated Iranian CD patients.

2. Materials and methods

2.1. Subjects and study design

In the current double-blinded, placebo-controlled intervention, 31 CD patients were randomly divided into two separate groups (based on block randomization); the placebo group (n = 16) and the probiotics group (n = 15). The randomization schedule was prepared by a trained investigator at coeliac Disease Department of Research Institute for Gastroenterology and Liver Diseases, Tehran using a computer-generated blocked random sequence. There was no significant difference in demographic and clinical characteristics between probiotics and placebo groups at baseline (P > 0.05). Flow diagram of the present study is shown in Fig. 1. An independent observer not taking part in the study performed labeling of study products. Symptomatic patients with good adherence to a strict GFD who were referred to the coeliac Disease Department in Research Institute for Gastroenterology and Liver Diseases, Tehran, Iran for a time period from October 2018 to June 2019 were recruited. Their GI and extra-GI symptoms were measured using the visual analogue scale (VAS). For determining fatigue state, feeling of weariness, listlessness, lack of energy, exhaustion, sleepiness and physical weakness during the days of study period were recorded for all subjects. All patients had some ongoing clinical symptoms and positive serum antibodies (tTGA and/or EMA) and were confirmed by histology according to the Marsh classification (Marsh II-III). Patients with other acute or chronic diseases, or any clinically significant disorder, pregnancy, and consumption of any medications or antibiotics at least one

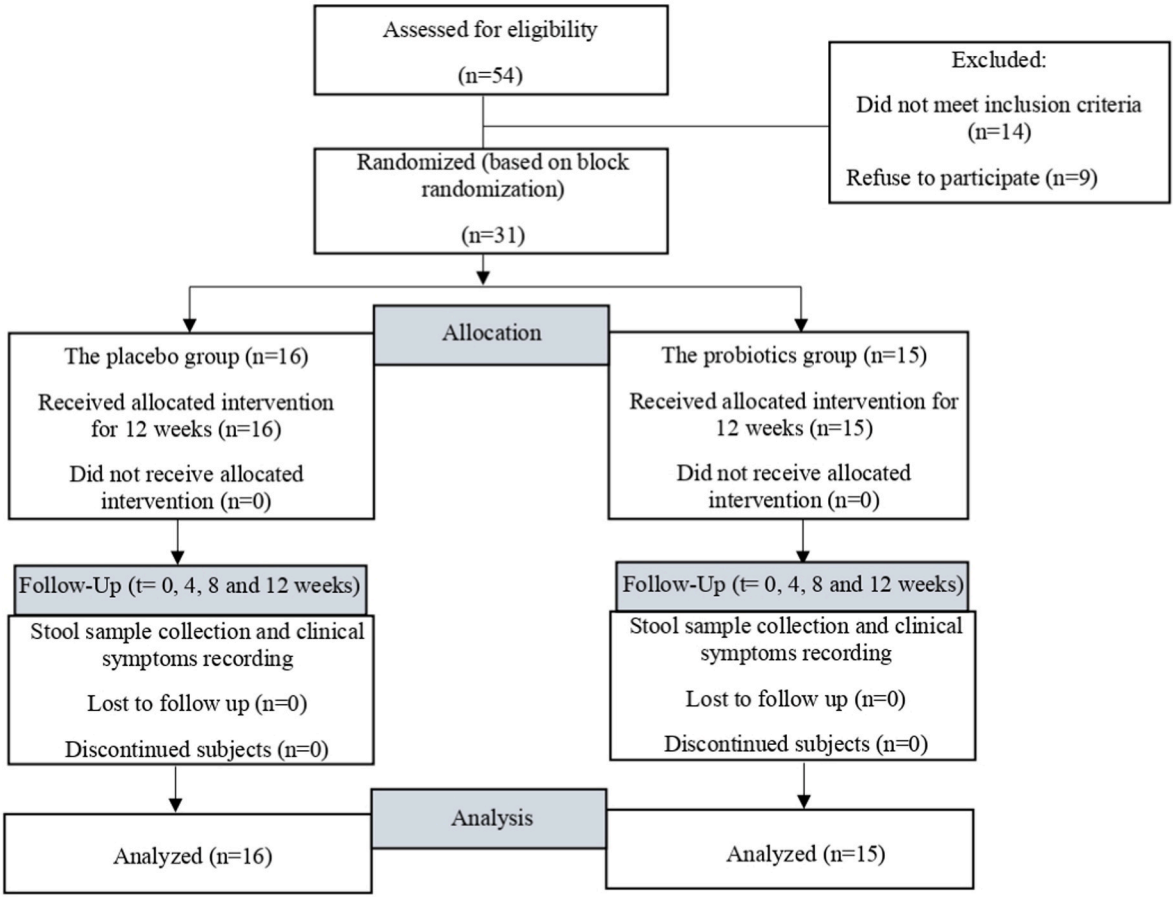

Fig. 1. Flow diagram of the current study.

month prior to the study were excluded. Demographic and clinical information of all participants including age, gender, weight, height, and Marsh grade were recorded using specific questionnaires.

The CD patients who met the inclusion criteria were blindly and randomly divided into two separate groups; the placebo group (n = 16) and the probiotics group (n = 15) randomized to receive the following formulation: placebo group, used 3 capsules (Zisttakhmir Co, Iran) per day containing only the excipient (fructo-oligosaccharide as prebiotic, lactose, Mg stearate, talc per day) with the same as treatment scheme, while probiotics group took three capsules (Zisttakhmir Co, Iran) containing a mixture of several bacterial strains including Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus bulgaricus, Bifidobacterium breve, Bifidobacterium longum, Streptococcus thermophiles (10^9 colony-forming units/capsule for each strain), three times a day before meals (breakfast, lunch, and dinner) for 12-weeks.

The study protocol was approved by the Ethical Review Committee of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences (Project No. IR.SBMU.RIGLD.REC.1395.114). All experiments were performed in accordance with relevant guidelines and regulations recommended by the institution and informed written consent was obtained from all subjects, and/or their legal guardians, prior to their inclusion in to study.

2.2. Collection of fecal samples

Fresh stool samples were collected from every subject enrolled in the placebo and probiotics groups at baseline (t = 0) and every four weeks at 4, 8 and 12 weeks. One stool sample was collected from the CD patients at each visit. Fresh stool samples (200 mg) were mixed with 1 mL sterile PBS (phosphate buffered saline, pH = 7.2) and homogenized by agitation using a vortex and aliquoted within 3 h of defecation. The aliquots were immediately frozen and stored at −80 °C in screw-capped cryovial tubes, until used for microbial DNA extraction.

2.3. Sample preparation and DNA extraction

Aliquots were used for DNA extraction using the FavorPrepTM Stool DNA Isolation Mini Kit (Favorgen® Biotech Corp., Pingtung, Taiwan) following the manufacturer's instructions. Briefly, 200 mg of feces were placed in a sterile, round-bottom 2 mL bead tube containing 300 μl of SDE1 buffer and 20 μl of proteinase K (10 mg/mL), and the rest of the protocol was followed as described by the manufacturer. The DNA concentration (ng) and its purity (absorbance/ratio at 260/280, 260/230) were determined spectrophotometrically using the NanoDrop®ND-1000 spectrophotometer (Thermo Scientific, USA), where pure DNA is defined as having a 260/280 absorbance ratio ranging between 1.7 and 2.0 and 260/230 ratio between 1.9 and 2.2. The integrity of genomic DNA was determined by visualizing approximately 200 ng of DNA on a solution of 1% agarose gel (w/v), containing 0.25 μg/μl of ethidium bromide (EtBr), and was run in 1X Tris-EDTA buffer at 100 V. The DNA aliquots were stored at −80 °C, until further analysis.

2.4. Microbiota analysis by quantitative real-time PCR

Real-time qPCR was used to quantify the different bacterial groups of the fecal microbiota using a set of universal, genus- and group-specific primers (Supplementary Table 1). Briefly, real-time PCR amplification was carried out in a Rotor-Gene® Q (Qiagen, Germany) real-time PCR system using SYBR Green chemistry. The real-time PCR reactions were performed in a total volume of 20 μl using BioFACT™ 2X Real-Time PCR Master Mix (For SYBR Green I, BIOFACT, South Korea) comprising 10 nM (each) forward and reverse primers and 2 μl of template DNA. The reaction conditions for amplification were 95 °C for 15 min and 40 cycles at 95 °C for 20 s, 56 °C for 30 s and 72 °C for 20 s which was followed by the melting curve step according to the manufacturer's instructions. The primer concentrations and thermocycling programs were optimized for each specific PCR reaction. The standard curve for 16S rRNA gene copy number quantification was performed by generating a series of 10-fold dilutions ranging from 101 to 1010 of 16S rRNA gene copies per reaction using the DNA of *Escherichia coli* BL21 strain. Melting curve analysis was also performed after the PCR to confirm the specificity of amplification. The amount of 16S rRNA gene copies of the specific bacterial groups in stool samples was determined by comparing the Ct values of samples with those of the standard curves. All of the reaction mixtures were run in triplicate.

2.5. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 21 (Armonk, NY: IBM Corp.). Differences in demographic characteristic measures between the study groups were assessed using the Chi-Square Pearson test. Comparisons between two different groups were made by the ANCOVA test with the t = 0 as the covariate to account for the initial discrepancy between the two groups. The results are expressed as mean ± standard deviation (SD). In all cases, P values less than 0.05 were considered statistically significant.

3. Results

Overall, 31 patients were included based on inclusion and exclusion criteria; from whom 15 received probiotic capsules and 16 received placebo. Table 1 shows the demographic characteristics of patients included in the present study. The mean age of the patients in both groups showed no statistically (ns) significant differences. As it is shown in Table 1, most of the patients in both groups were classified into Marsh 3. The results showed ns significant differences regarding the Marsh classification in both groups. The mean baseline of body weight in the probiotics group was 60 ± 14 kg, in the placebo group was 57 ± 25 kg, and it rose to 67 ± 8 and 61 ± 30 kg, respectively at the end of the study (Table 2).

Following the study protocol and in terms of the clinical symptoms, at the beginning of the study, both probiotics and placebo groups shared almost similar rates of each symptom. The most common clinical symptoms were fatigue and bloating, respectively. At baseline, ns differences were found in the severity of studied symptoms between the two groups (Table 3). However, during the follow-up at weeks 4, 8 and 12, symptoms were reduced in both groups with higher improvement in the probiotics group. The severity of fatigue was significantly reduced at weeks 8 and 12 of the intervention in the probiotics group compared to the placebo (Fig. 2).

In terms of microbiota analysis, the results showed that at baseline *Firmicutes* followed by *Clostridium* cluster I were the most predominant bacteria in CD patients. Results obtained from microbiota analysis after the intervention showed that the administration of the probiotics led to

Table 1
Baseline demographics of the study participants. Differences in demographic characteristics between the two groups were assessed using the Pearson's chi-squared test.

Demographic factors		Study groups		*P* value
		Placebo (n = 16)	Probiotics (n = 15)	
		Mean ± SE	Mean ± SE	
Age (years)		32 ± 15	39.5 ± 20	0.25
Males		8 (50%)	7 (46.7%)	0.83
Females		8 (50%)	8 (53.3%)	
Baseline height (cm)		156 ± 29	164 ± 13	0.35
Baseline weight (kg)		57 ± 25	60 ± 14	0.67
Marsh classification	March 2	3 (18.6%)	0	0.57
	Marsh 3	13 (81.2%)	15 (100%)	

Table 2
Trend of coeliac disease patients' weight changes in probiotics and placebo groups during the study period.

Weight (kg)	Study groups		*P* value
	Placebo (n = 16)	Probiotics (n = 15)	
	Mean ± SE	Mean ± SE	
Baseline	57 ± 25	60 ± 14	0.67
Week 4th	58 ± 29	59 ± 16	0.86
Week 8th	58 ± 32	66 ± 8	0.42
Week 12th	61 ± 30	67 ± 8	0.61

Table 3
Baseline clinical symptoms of the study participants.

Clinical symptoms	Placebo (n = 16)	Probiotics (n = 15)	*P* value
Abdominal pain	9 (56%)	6 (40%)	0.38
Bloating	11 (69%)	13 (87%)	0.25
Fatigue	15 (94%)	11 (73%)	0.13
Gas feeling	9 (56%)	10 (67%)	0.57
Heartburn	10 (63%)	10 (67%)	0.82
Muscle pain	8 (50%)	8 (53%)	0.86

an increase in the abundance of *Bacteroidetes*, *Clostridium* cluster I, *Enterobacteriaceae*, *Staphylococcus* spp., *Bifidobacterium* spp., *Lactobacillus* spp. and *Firmicutes*. Comparisons between the study groups showed that the numbers of *Firmicutes*, *Bacteroidetes*, *Bifidobacterium* spp., *Lactobacillus* spp. and *Enterobacteriaceae* were increased in the probiotics group. The rate of *Clostridium* cluster I, *Bacteroidetes*, *Enterobacteriaceae*, *Lactobacillus* spp., *Bifidobacterium* spp. and *Firmicutes* except for *Staphylococcus* spp. was higher in the probiotics group compared to placebo at the end of the intervention period, but these differences were ns significant ($P > 0.05$) (Fig. 3). No correlation was observed between the relief of clinical symptoms and microbiota composition using the Kruskal-Wallis test ($P > 0.05$).

4. Discussion

Change in the composition of gut microbiota is an important environmental factor that has contributed to autoimmune inflammatory disorders of the human intestines including CD [5,26,27]. It is known that there are relatively heterogeneous causes of persistent symptoms in the majority of CD patients who are on a GFD, and in particular, alterations in the gut microbiota composition can be one of them [28–31]. In this regard, Wacklin et al. [18] showed that the composition of the duodenal microbiota in symptomatic CD patients is different from asymptomatic patients. These researchers suggested that an imbalanced gut microbiota, known as gut dysbiosis, may be one of the causes of persistent GI symptoms in CD patients on a GFD.

A substantial number of treated CD patients with persistent symptoms like fatigue, bloating, abdominal pain, heartburn, gas feeling and muscle pain, referred to our clinic, and we investigated the impact of probiotic supplementation on their clinical symptoms improvement and its effects on the intestinal microbiota composition. Our study showed a decrease in the severity of clinical symptoms after the administration of a probiotic mixture, including *Lactobacillus* spp., *Bifidobacterium* spp. and *S. thermophilus*. Patients in the probiotics group reported less severity of clinical manifestations (fatigue, muscle pain, bloating, and gas feeling) compared to the placebo group. We observed a significant reduction in fatigue at weeks 8 and 12 among patients who received probiotic supplementation. In fact, the human gut microbiota consists of different microorganisms including Gram-positive and Gram-negative bacteria [32]. In most CD patients, the number of Gram-negative bacteria including *Bacteroides*, *E. coli*, and *Enterobacteriaceae* is increased, and the number of Gram-positive bacteria including *Bifidobacterium*, *Streptococcus*, and *Lactobacillus* spp. is decreased compared to healthy subjects [9,33–36]. Administering probiotics by inducing the increase of Gram-positive bacteria and restoring the intestinal microbial balance may has a crucial role in alleviating intestinal inflammation leading to relieving clinical symptoms of patients with intestinal inflammatory diseases like CD. Previous studies have also shown an improvement in overall clinical symptoms in CD patients after probiotic supplementation [37,38]. In a retrospective, double-blind, randomized placebo-controlled study, Francavilla et al. investigated the effect of a mixture of five strains of *lactic acid bacteria* and *Bifidobacterium* spp. on CD patients with irritable bowel syndrome (IBS)-type symptoms on strict GFD and found that six weeks of treatment with probiotics were able to decline the severity of IBS-like symptoms in CD patients [38,39]. They concluded that improvement in the severity of IBS-type symptoms was associated with a modification in gut microbiota, characterized by an increase in the abundance of *Bifidobacteria*. Additionally, an improvement in some clinical symptoms after the 3 weeks of treatment by *B. infantis* was reported in another study by Smecuol et al. [37]. On the contrary, after the administration of VSL#3, a well-known multi-strain probiotic with eight different bacterial strains containing *Lactobacilli* (*L. casei, L. plantarum, L. acidophilus,* and *L. delbrueckii*), *Bifidobacteria* (*B. longum, B. breve,* and *B. infantis*), and *Streptococcus* (*S. salivarius*), Harnett et al. found no clinically significant improvement in symptoms between treatment and placebo groups [39].

Moreover, the present results showed a relative rise in all studied microbial taxa in treated CD patients after probiotic administration. As stated before, our probiotic capsule contained a mixture of *Lactobacillus* spp., *Bifidobacterium* spp., and *Streptococcus* spp., which can help in improving the stability of gut microbiota and protect against gastrointestinal disorders [40]. In fact, alteration of the intestinal microbiome leads to uncontrolled inflammation in the intestinal mucosa and reverting that to the original state by utilizing probiotic supplements might help restoring the mucosal integrity of patients with intestinal disorders [41]. Moreover, the proportion of *Firmicutes* and *Bacteroidetes* was increased after a 12-week probiotic treatment. A similar study evaluated the effects of three months' administration of a probiotic supplement based on two *B. breve* strains (B632 and BR03) on 40 children with CD and demonstrated an increase in *Firmicutes* abundance and restoration of the physiological *Firmicutes/Bacteroidetes* ratio [28]. Primec et al. also showed an increase in *Firmicutes* after three months of administration of *B. breve* strains B632 and BRO3 in children with CD under GFD [42]. In our study, administration of the probiotics led to an increase in proportions of *Bifidobacterium* spp. and *Lactobacillus* spp. Quagliariello et al. [28]. demonstrated a slight increase in counts of *Bifidobacterium* spp. in the intestinal microbiota of CD patients after treatment with probiotics containing *B. breve*. Similarly, our findings showed that intake of probiotics for 12 weeks increased the proportion of fecal *Bifidobacterium* spp., *Lactobacillus* spp., *Staphylococcus* spp., *Bacteroidetes*, and *Enterobacteriaceae* in CD patients. Olivares et al. [43] investigated the effects of *B. longum* CECT 7347 on intestinal microbiota composition in 33 children with newly diagnosed CD. Based on their results a GFD plus *B. longum* CECT 7347 administration for three months tends to cause reductions in total bacteria and the gene copy numbers of the *Bacteroides fragilis* group [43]. Besides probiotic dosage, this controversy may also arise from differences in sample size, target population, type of study, duration of treatment and differences in probiotic strains.

Firmicutes was the most predominant bacteria in our studied patients. Bodkhe et al. also observed that *Firmicutes* was one of the dominant bacterial phyla in the duodenal microbiota of CD patients on GFD [35]. Another study on the gut microbiota composition of CD patients showed that *Firmicutes*, Proteobacteria, and *Bacteroidetes* were the major bacterial phyla of the upper small intestine in children and adults, including healthy subjects, untreated CD, and treated CD patients [34]. Furthermore, it was demonstrated that microbial richness was reduced in treated CD patients with persistent symptoms following a higher relative abundance of Proteobacteria and a lower abundance of *Bacteroidetes* and

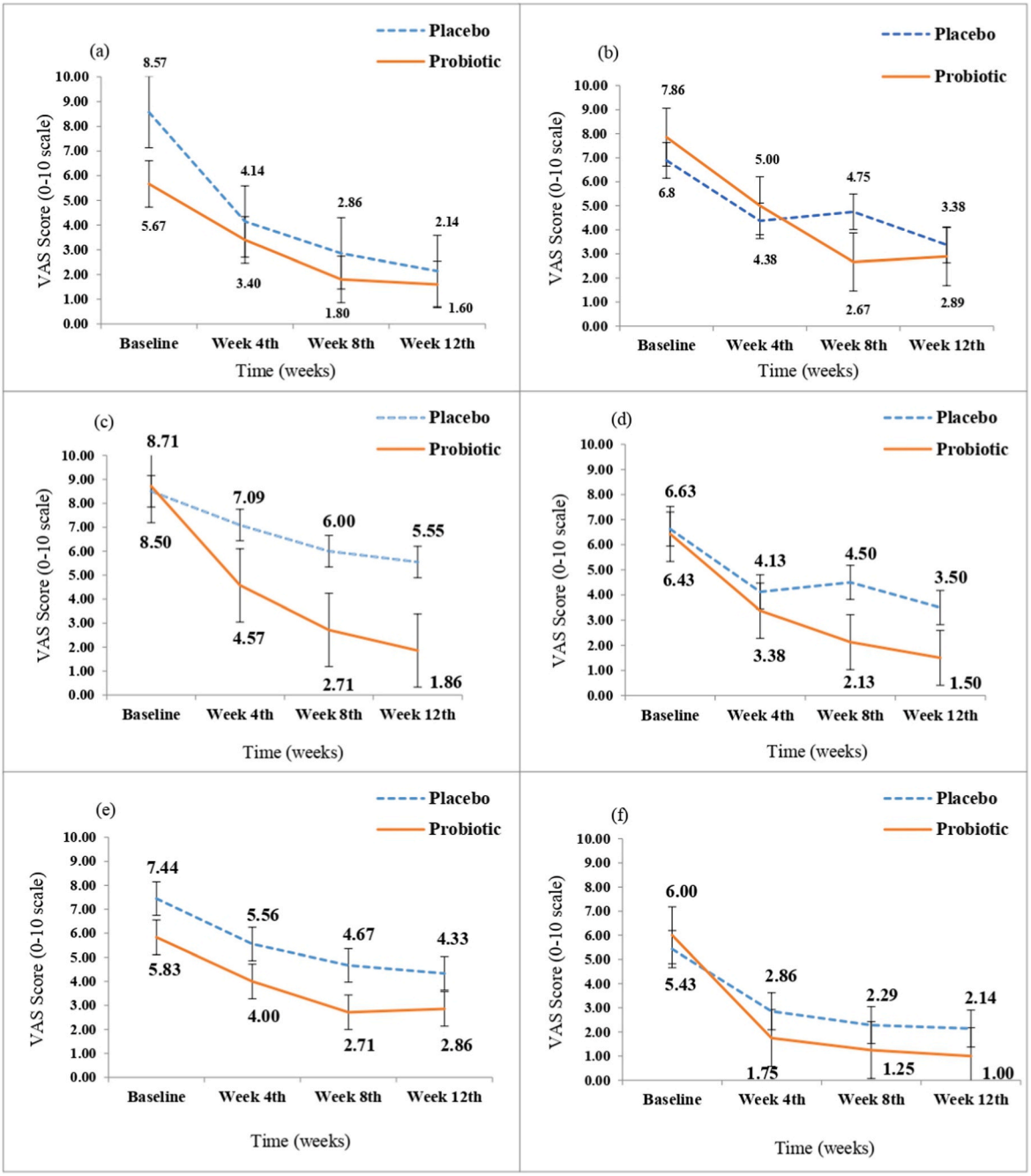

Fig. 2. Trends of GI and extra-GI symptoms during 12 weeks of intervention with probiotics represented by the visual analogue scale in the placebo group (dotted line) and the probiotics group (solid line). (a) Abdominal pain; (b) bloating; (c) fatigue; (d) feeling of gas; (e) heartburn; (f) muscle pain. Variations at the baseline for each symptom were considered as covariates for statistical analysis (comparisons between two studied groups were made by the ANCOVA test).

Firmicutes while adhering to a strict GFD [18]. Our previous study also showed that meat and bean consumptions had an inverse effect on the abundance of beneficial bacteria like *Firmicutes* and *Lactobacillus* in CD patients, which means that paying attention to the food intake of CD patients is of great importance [44].

The present study had some limitations. First, this study had a small sample size and was conducted at a single center in Tehran, Iran. Second, in this study, we only analyzed the fecal gut microbiota and not the mucosal-associated microbiota, which are potentially exposed to different factors and may lead us to conflict results. Third, we used specific primer sequences for a select bacterial taxa, which could not enable us to identify the whole bacterial species present in fecal samples. Thus, it is highly recommended for future studies to employ a universal 16S rRNA-based sequencing.

5. Conclusions

Taken together, further studies using larger cohorts are required to validate whether the administration of probiotic strains could completely restore the gut microbiota composition and ameliorate the severity of symptoms in Iranian CD patients.

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Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti

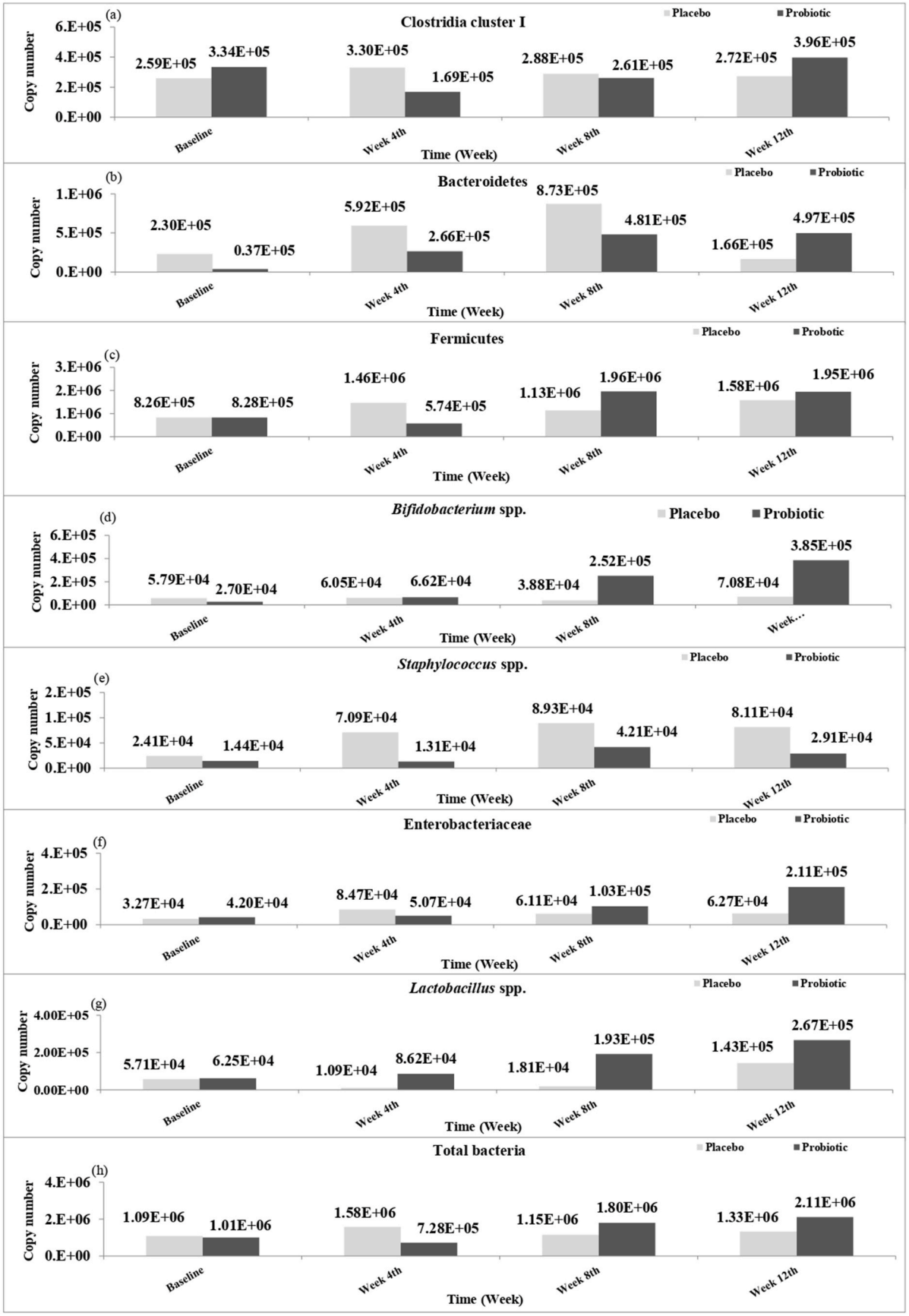

Fig. 3. Changes in the gut microbiota copy numbers obtained from amplification of 16S rRNA gene copies using real-time qPCR and standard curve analysis between probiotics group (dark grey) and placebo group (light grey) during 12 weeks of intervention. Independent *t*-test was used to determine significant difference. (a) *Clostridium* cluster I; (b) *Bacteroidetes*; (c) *Fermicutes*; (d) *Bifidobacterium* spp.; (e) *Staphylococcus* spp.; (f) *Enterobacteriaceae*, (g) *Lactobacillus* spp.; (h) total bacteria.

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Author contributions and consent for publication

Conceptualization: M.R.N., A.Y., and H.D.; Methodology: M.S.K., and A.Y.; Validation: M.R.N., A.Y., and H.D.; Formal analysis: A.H., A.Y., L. R., and M.R.N.; Original draft preparation: M.S.K.; Review and critical editing: A.Y., M.R.N., and L.R. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethical Review Committee of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences (Project No. IR.SBMU.RIGLD.REC.1395.114). All experiments were performed in accordance with relevant guidelines and regulations recommended by the institution and informed written consent was obtained from all subjects, and/or their legal guardians, prior to their inclusion in to study.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.conctc.2023.101201.

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