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Placebo-resistant gut bacteria: *Akkermansia muciniphila* spp. and Familial Mediterranean fever disease

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Introduction: Despite numerous investigations into the impact of drugs/probiotics on the gut microbiota composition in Familial Mediterranean Fever (FMF) patients, the question as to whether there exists a significant bacterial diversity(ies) independent of the placebo effect that can be reliably considered in clinical and nutritional trials remains unresolved.

Methods: This study represents the in augural analysis of the placebo's influence on the gut microbiota of both healthy individuals and FMF afflicted men, utilizing previously collected data from PhyloChip™ DNA microarray experiments. A total of 15 healthy and 15 FMF male volunteers, aged 18 to 50, participated in this partially randomized placebo trial, which is accessible through the GEO Series accession number GSE111835.

Results and Discussion: Key findings from current investigations include *i.* the anticipated divergence in gut bacteria resistance to placebo between healthy and FMF individuals, *ii.* the minor impact of placebo on gut bacterial diversities in healthy individuals, with *Enterobacteriaceae* diversities identified as placebo-resistant among "healthy" gut bacteria, and *iii.* the comprehensive influence of placebo on all bacterial phyla in the gut microbiome of FMF patients, extending to nearly all bacterial genera, except for the resilience of gut *Akkermansia muciniphila* spp. to placebo in FMF patients. This study underscores the susceptibility of *Faecalibacterium*, *Blautia*, and *Clostridium* genera to placebo. Consequently, this investigation holds significance for the proper design of placebo-controlled trials and establishes a foundation for further exploration of the gut-brain axis. Furthermore, it contributes valuable insights to discussions regarding proposals for probiotic therapies, particularly focusing on *Faecalibacterium* spp., *Blautia* spp., and *Clostridium* spp.

KEYWORDS

placebo, male patients, microbiome, *Akkermansia muciniphila*, familial Mediterranean fever, *Enterobacteriaceae* spp., *Faecalibacterium*, *Blautia*

Introduction

Clinical trials involving healthy controls face challenges in both subject recruitment and result interpretation (Johnson et al., 2016). Additionally, the orchestration of placebo-controlled trials is a complex process (Howick, 2017), particularly in developing countries (Lepage et al., 2023; Kim et al., 2023). The success of clinical trials is influenced by diverse factors (Kupersmith and Jette, 2023; Jacobsen et al., 2023; Feldman et al., 2022), and despite the abundance of such trials, there remains a need to elucidate placebo effects in both healthy individuals and patients, particularly in studies related to the gut microbiota (Kleine-Borgmann et al., 2023).

Familial Mediterranean Fever (FMF) is a monogenic autosomal recessive autoinflammatory disorder resulting from mutations in the MEFV gene. The disease is characterized by inflammatory episodes affecting serous membranes, leading to periodic fevers and pain (Pepoyan A. et al., 2015; Pepoyan AZ. et al., 2015; Talerico et al., 2020; Bhatt and Cascella, 2023; Gallego et al., 2023; Zadeh et al., 2011). In the context of FMF, placebos are utilized to assess the effects of various drugs and functional foods, including probiotics [beneficial bacteria for humans (Pepoyan and Trchounian, 2009; Pepoyan et al., 2018b)/animals/plants (Manvelyan et al., 2023; Pepoyan et al., 2019a)]. While it is hypothesized that diets rich in antioxidants and supplements with anti-inflammatory properties may partially alleviate FMF symptoms and enhance the well-being of patients, research findings in this realm are contentious (Mansueto et al., 2022; Damián et al., 2022; Mazzantini et al., 2021). The number of clinical trials on FMF is notably high, given the monogenic nature of the disease (Welzel et al., 2021; Tsaturyan et al., 2023; Pepoyan et al., 2018a; Ataya et al., 2023).

The composition of microbiomes is influenced by various factors, including host genetics (Lighthouse et al., 2004; Qin et al., 2022; Boccuto et al., 2023; Pepoyan et al., 2020a), prevailing diseases (Lighthouse et al., 2004; Qin et al., 2022; Boccuto et al., 2023; Pepoyan et al., 2020a), environmental conditions (Berg et al., 2020; Zengler et al., 2019; Kozhakhmetov et al., 2023), and the inherent self-assembly properties of microbes (van Leeuwen et al., 2023; Hovnanyan et al., 2015).

Numerous pieces of evidence suggest a nuanced interplay between microbiota perturbations and the phenotypic expressions of FMF, with the complexity of this relationship influenced by both genetic and environmental factors. The modulation of gut microbiota, encompassing the investigation of probiotic treatments, holds promise for advancing our understanding and management of FMF (Di Ciaula et al., 2020).

Nevertheless, there is ongoing controversy regarding the ability of probiotic treatment to alter the composition of the host microbiota. The health benefits associated with probiotics may arise from the metabolites produced by the bacteria and their interactions with the host's immune system (Singh et al., 2023). Notably, probiotics have shown the ability to influence gene expression, exerting potential anti-inflammatory effects within the gut microbiota without inducing changes in composition (Ng et al., 2023).

In Armenia, a significant number of male patients with Familial Mediterranean Fever (FMF) has been reported (Tsaturyan et al., 2023). Additionally, there are documented impairments in the host-gut microbiota relationship in FMF disease (Tsaturyan et al., 2023). Moreover, the impact of the probiotic *Lactobacillus acidophilus* strain INMIA 9602 Er 317/402 on the gut microbiota composition of male FMF patients was demonstrated through a placebo/probiotic comparative analysis (Pepoyan et al., 2018a). However, existing data and analyses concerning the effects of placebo on the gut microbiota are limited and do not provide a comprehensive understanding of the placebo's influence on the overall bacterial composition of the gut microbiota.

The primary objective of this study is to assess the effects of a placebo on the composition of the gut microbiota in male FMF patients. The central research question aims to determine whether there exists a significant diversity of bacteria independent of the placebo effect that can be directly utilized in clinical and nutritional trials.

Materials and methods

In this study, for the first time, the effect of placebo on the gut microbiota of healthy and FMF men was fully analyzed by leveraging the prior PhyloChip™ DNA-microarray-based data (Tsaturyan et al., 2023; Pepoyan et al., 2018a). Healthy and FMF male volunteers (15/15) aged 18 to 50 took part in this partially randomized placebo trial accessible through GEO Series accession number GSE111835 (Tsaturyan et al., 2023; Pepoyan et al., 2018a) in which the participant took an empty capsule twice daily as a placebo for 1 month. The patients also took their main drug, 1 mg colchicine, as usual. All patients' diagnoses were confirmed by genetic analysis. None of the study participants had been treated with antibiotics, probiotics, hormones, or chemotherapeutic agents during the month leading up to the study. The duration of the colchicine treatment by patients was more than 7 years. Patients in the acute phase were not included in the study.

Standardization protocols of DNA isolation were implemented to enhance the reliability and comparability of gut microbiome analyses. Samples' metadata, such as date, time, and participant information, were properly documented and stored. Stool samples collected by volunteer subjects in sterile plastic bags and transported to the laboratory were studied. In order to obtain optimal yield and quality of DNA, both ZR Fecal DNA MiniPrep™ (Zymo Research Corp., Irvine, CA, USA) and Ultraclean® Fecal DNA Isolation (MoBio Laboratories Inc., Carlsbad, CA, USA) commercially available kits were used to isolate total DNA. Chosen DNA extraction kits have been validated for fecal samples. To ensure that the entire sample is homogenized consistently to obtain representative microbial DNA, the bead beating method (FastPrep-24, MP Biomedicals, USA) was used. The extracted DNA was quantified using an absorbance-based method (NanoDrop Microvolume Spectrophotometers, Thermo Fisher, USA). DNA quality was assessed using gel electrophoresis. gDNA was extracted/amplified (16S rRNA gene) from fecal materials frozen at -80°C. The primer sequences used for microarrays and 16S

rRNA clone libraries were: 27f.jgi (Bacteria-specific) 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492r.jgi (Bacteria/Archaea-specific) 5′-GGTTACCTTGTTACGACTT-3′.

Bacterial communities were identified using a third-generation, culture-independent, high-density DNA microarray analysis (PhyloChip™; Affymetrix, Santa Clara, CA, USA), according to the investigations described previously (Tsaturyan et al., 2023; Pepoyan et al., 2018a).

This method also enables the estimation of differences in the relative abundance of bacterial taxa based on differences in their hybridization intensities (Kellogg et al., 2013).

In this study, pharmaceutical-grade empty hard-gelatin capsules sourced from Vitamax E, LLC in Yerevan, Armenia, were employed. These gelatin capsules, recognized for their swift absorption in the gastrointestinal tract and their lack of side effects, adhere to GMP, USP, and SP standards. The study participants were unaware that the placebo capsules were empty.

Student's t-test and Mann-Whitney test were used for statistical analyses. $P < 0.05$ was considered statistically significant. Multibase 2015 Excel Add-in program (NumericalDynamics, Tokyo, Japan) was also used in the prior studies (Tsaturyan et al., 2023; Pepoyan et al., 2018a).

Results

Comparative analysis of gut microbiota composition of non-FMF and FMF men before and after the placebo administration: bacterial diversities

A comparative analysis of gut microbiota composition was conducted for non-FMF and FMF men before and after placebo administration, focusing on bacterial diversities. The evaluation covered 18,725 bacterial Operational Taxonomic Units (OTUs) to identify variations in gut bacterial diversities.

In the non-FMF male group, the analysis revealed that 140 OTUs exhibited statistically significant differences after the administration of the placebo ($P < 0.05$). The altered bacteria primarily belonged to the phyla *Firmicutes* (78 OTUs), *Bacteroidetes* (17 OTUs), *Proteobacteria* (16 OTUs), and *Tenericutes* (9 OTUs) (Table 1).

Similarly, in the FMF male group after placebo administration, 7,560 OTUs were altered in the gut microbiota of patients. Among these, 4,777 belonged to *Firmicutes*, 1,360 to *Proteobacteria*, 350 to *Bacteroidetes*, 119 to *Tenericutes*, 23 to *Actinobacteria*, and the remainder to other bacterial phyla ($P < 0.05$) (Table 1).

TABLE 1 Number of different OTUs after taking the placebo: bacterial phyla*.

Bacterial phyla	Healthy (non-FMF men) OTUs	FMF men OTUs
Firmicutes	78 (55.71)	4,777 (63.18)
Bacteroidetes	17 (12.14)	350 (4.62)
Proteobacteria	16 (11.43)	1,360 (17.99)
Tenericutes	9 (6.43)	119 (1.57)
Actinobacteria	5 (3.57)	23 (0.304)
Other	15 (10.72)	929 (12.34)
Sum	140	7,560

*The impact of the placebo on 18,725 bacterial OTUs was evaluated; $P < 0.05$.
In parentheses- OTUs percentages.
FMF, familial Mediterranean fever.
OTUs, operational taxonomic units.

Impact of placebo: differences in bacterial diversities of *Firmicutes*

The impact of placebo on bacterial diversities within the *Firmicutes* phylum revealed distinctive patterns in non-FMF and FMF men.

For non-FMF men, *Firmicutes* OTUs constituted 55.71% of all different bacterial OTUs, with prominent differences in the order *Clostridiales* (66 OTUs). These differences primarily comprised OTUs from families *Clostridiaceae* (6 OTUs), *Lachnospiraceae* (16 OTUs), and *Ruminococcaceae* (40 OTUs, mostly from an unclassified genus, and 7 OTUs from the genus *Faecalibacterium*). Additionally, 7 OTUs were from the order *Lactobacillales*, and 5 OTUs were from *Bacillales* (Tables 2, 3).

For FMF men, *Firmicutes* OTUs constituted 63.18% of all different bacterial OTUs, with predominant differences in the order *Clostridiales* (83%) (Table 2).

Analysis from Table 3 indicates that the families *Ruminococcaceae* and *Lachnospiraceae* were particularly susceptible to placebo within the *Firmicutes* phylum. Following placebo administration, the numbers of altered OTUs from the families *Ruminococcaceae* for non-FMF men and FMF men were 40 (63.61%) and 1365 (35.39%), respectively. Similarly, the numbers of altered OTUs from the families *Lachnospiraceae* for non-FMF men and FMF men were 16 (26.51%) and 2086 (54.96%), respectively (Table 3).

Impact of placebo: differences in bacterial diversities of *Bacteroidetes*

The impact of the placebo on the differences in bacterial diversities within the phylum *Bacteroidetes* was examined, highlighting distinctions between non-FMF and FMF men.

TABLE 2 Number of different OTUs after taking the placebo: *Firmicutes**.

Phylum Firmicutes: orders	Healthy (non-FMF men) OTUs	FMF men OTUs
Clostridiales	66 (85)	3,969 (83)
Lactobacillales	7 (9)	500 (10.99)
Bacillales	5 (6)	265 (5.99)
Sum	78	4,777

*The impact of the placebo on 18,725 bacterial OTUs was evaluated; $P < 0.05$.
In parentheses- OTUs percentages.
FMF, familial Mediterranean fever.
OTUs, operational taxonomic units.

TABLE 3 Number of different OTUs after taking the placebo: *Clostridiales**.

Order Clostridiales: families	Healthy (non-FMF men) OTU	FMF men OTU
Ruminococcaceae	40 (63.61) (mostly from the unclassified genus, and 7 OTUs from the genus *Faecalibacterium*)	1,365 (35.39)
Lachnospiraceae	16 (26.51)	2,086 (54.96)
Clostridiaceae	6 (9.88)	361 (9.65)
Sum	66	3,969

*The impact of the placebo on 18,725 bacterial OTUs was evaluated; P < 0.05.
In parentheses- OTUs percentages.
FMF, familial Mediterranean fever.
OTUs, operational taxonomic units.

In non-FMF men, among *Bacteroidetes* OTUs, which comprised 12.14% of all different bacterial OTUs (Table 1), the prominent differences were associated with OTUs of the *Bacteroidia* class, making up 70.59% of the phylum (Table 4).

For FMF men, among *Bacteroidetes* OTUs, accounting for 4.62% of all different bacterial OTUs (Table 1), the significant differences were linked to OTUs of the *Bacteroidia* class, constituting 79.44% of the phylum (Table 4). Following placebo administration, OTUs from the families of *Prevotellaceae* (30.32%), *Bacteroidaceae* (28.88%), and *Rikenellaceae*II (23.10%) emerged as the quantitatively dominant different bacterial families within the class (Table 4).

Impact of placebo: differences in bacterial diversities of *Proteobacteria*

In non-FMF men, among *Proteobacteria* OTUs, constituting 11.43% of all different bacterial OTUs (Table 1), the predominant differences were associated with OTUs from the following classes: 43.75% *Betaproteobacteria*, 25% *Alphaproteobacteria*, and 25% *Gammaproteobacteria*. It is noteworthy that all different *Gammaproteobacteria* OTUs belonged to the genus *Pseudomonas* ($P < 0.05$) (Table 5). Interestingly, there were no placebo-induced alterations in bacterial diversities within *Enterobacteriaceae*.

For FMF men, among *Proteobacteria* OTUs, accounting for 17.99% of all different bacterial OTUs (Table 1), the predominant differences were related to OTUs of the *Enterobacteriaceae*, constituting 39.41% of the different *Proteobacteria* ($P < 0.05$) (Table 5).

TABLE 4 Number of different OTUs after taking the placebo: *Bacteroidetes* *.

Bacteroidetes: classes	Healthy (non-FMF people)	FMF people
Bacteroidia	12 (70.59) (prevailed family: *RikenellaceaeII*- 8 OTUs)	277 (79.44) (prevailed families: *Prevotellaceae*: 84 OTUs *Bacteroidaceae*- 80 OTUs *RikenellaceaeII*- 64 OTUs *Porphyromonadaceae*- 24 OTUs)
Flavobacteria	1 (5.88)	37 (11.57)
Sphingobacteria	4 (23.53)	32 (8.99)
Sum	17	350

*The impact of the placebo on 18,725 bacterial OTUs was evaluated; P < 0.05.
In parentheses- OTUs percentages.
FMF, familial Mediterranean fever.
OTUs, operational taxonomic units.

Impact of placebo on hybridization scores of different bacterial diversities

In line with the substantial number of distinct OTUs recorded in FMF disease (Table 1), the hybridization scores of bacterial OTUs in FMF individuals after placebo were significantly greater than those of non-FMF individuals:

— *Ruminococcus* spp.: 16 100,866 ± 3 309,224 vs. 531,574 ± 8,309.14
— *Lachnospiraceae* spp.: 17 861,159 ± 4 625,717.4 vs. 200,364.3 ± 3,952.3
— representatives from the *Bacteroidia*: 1 874,301 ± 554,143.2 vs. 146,473.8 ± 8,583.8.

According to the results of the hybridization scores, in the non-FMF male subjects, the placebo produced quantitative changes in the altered main bacterial diversities, which were not observed in the FMF male subjects. There was an increase in *Ruminococcus* spp (513,870.3 ± 10,018.57 vs. 531,574 ± 8,309.14; $P < 0.05$) and *Lachnospiraceae* spp. (194,678 ± 4,802.6 vs. 200,364.3 ± 3,952.3, $P < 0.05$), as well as representatives of *Bacteroidia* after the placebo intake in non-FMF men (Figure 1).

TABLE 5 Number of different OTUs after taking the placebo: Proteobacteria*.

Proteobacteria: *families*	Healthy (non-FMF people) OTUs	FMF people OTUs
Enterobacteriaceae	0	536 (39.41)
Aquabacteriaceae	2 (12.5)	175 (12.87)
Comamonadaceae	2 (12.5)	81 (5.6)
Pseudomonadaceae	4 (25.0)	76 (5.59)
other	8 (50)	492 (35.83)
Sum	16	1,360

*The impact of the placebo on 18,725 bacterial OTUs was evaluated; P < 0.05, and the diversities with comparatively large numbers were considered.
In parentheses- OTUs percentages.
FMF, familial Mediterranean fever.
OTUs, operational taxonomic units.

Overlapping gut bacterial diversities in non-FMF and FMF men after the placebo administration (number of OTUs)

After the placebo administration, a total of 54 overlapping gut bacterial diversities were identified from the pool of 18,725 bacterial OTUs in both non-FMF and FMF men. These 54 OTUs were primarily affiliated with the following families:

— *Lachnospiraceae* (12 OTUs) (Figure 2).

— *Ruminococcaceae* (10 OTUs) (Figure 3).

Differences in order *Clostridiales* diversities in non-FMF men after the placebo administration

Differences in order *Clostridiales* diversities were noted in non-FMF men after the placebo administration. According to Table 3, the number of distinct OTUs for the families *Ruminococcaceae, Lachnospiraceae*, and *Clostridiaceae* spp. after the placebo intake was 40, 16, and 6, respectively, for non-FMF men. When comparing this data with the information on "overlapping gut bacterial

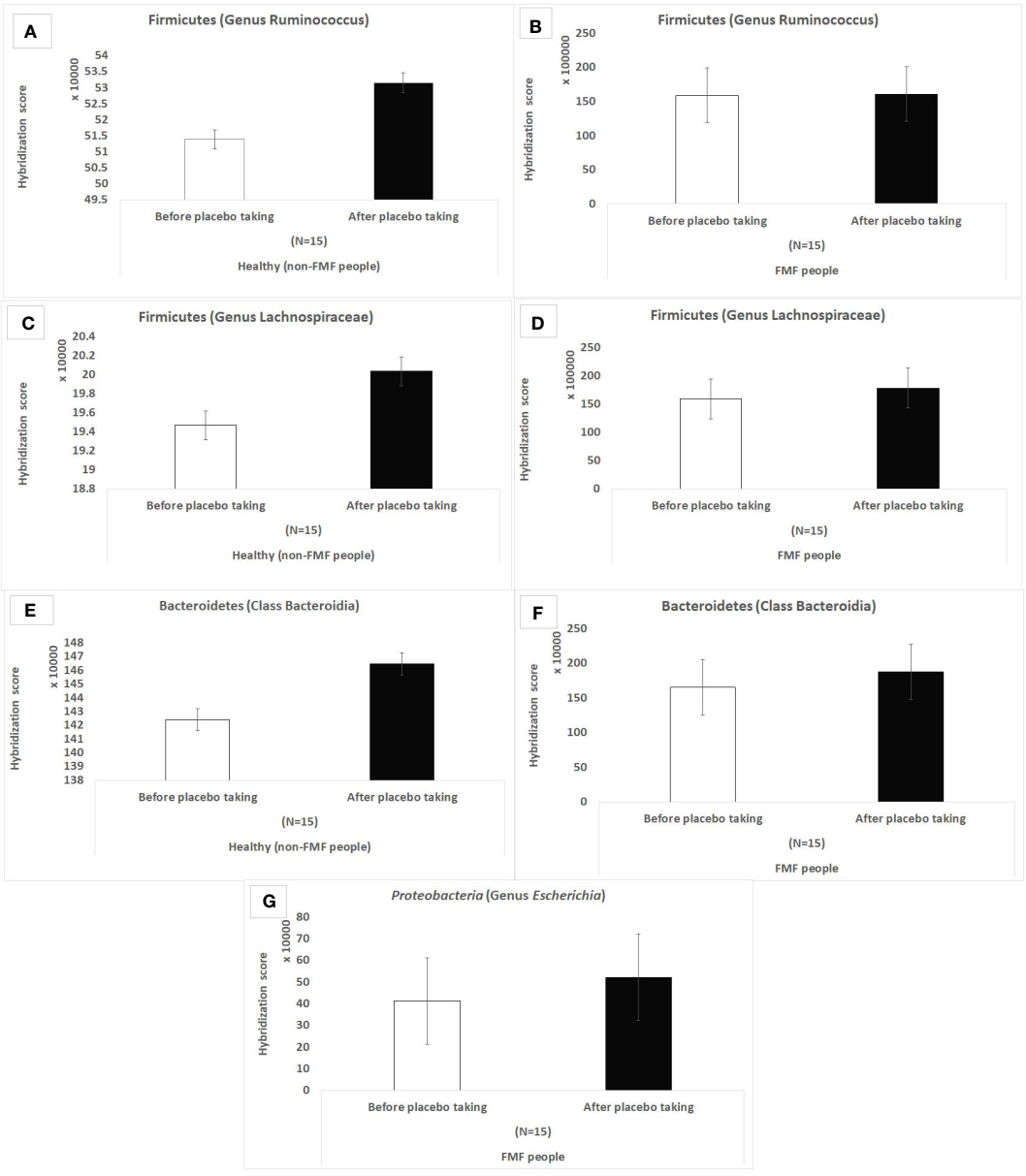

FIGURE 1
Hybridization scores of altered gut bacterial diversities in non-FMF and FMF men after the placebo administration. The impact of the placebo on 18,725 bacterial OTUs was evaluated; $P < 0.0001$. FMF – Familial Mediterranean fever. OTUs – operational taxonomic units. **(A)** Hybridization score of *Ruminococcus* OTUs after placebo in non-FMF men. **(B)** Hybridization score of *Ruminococcus* OTUs after placebo in FMF men. **(C)** Hybridization score of *Lachnospiraceae* OTUs after placebo in non-FMF men. **(D)** Hybridization score of *Lachnospiraceae* OTUs after placebo in FMF men. **(E)** Hybridization score of *Bacteroidia* OTUs after placebo in non-FMF men. **(F)** Hybridization score of *Bacteroidia* OTUs after placebo in FMF men. **(G)** Hybridization score of *Escherichia* OTUs after placebo in FMF men.

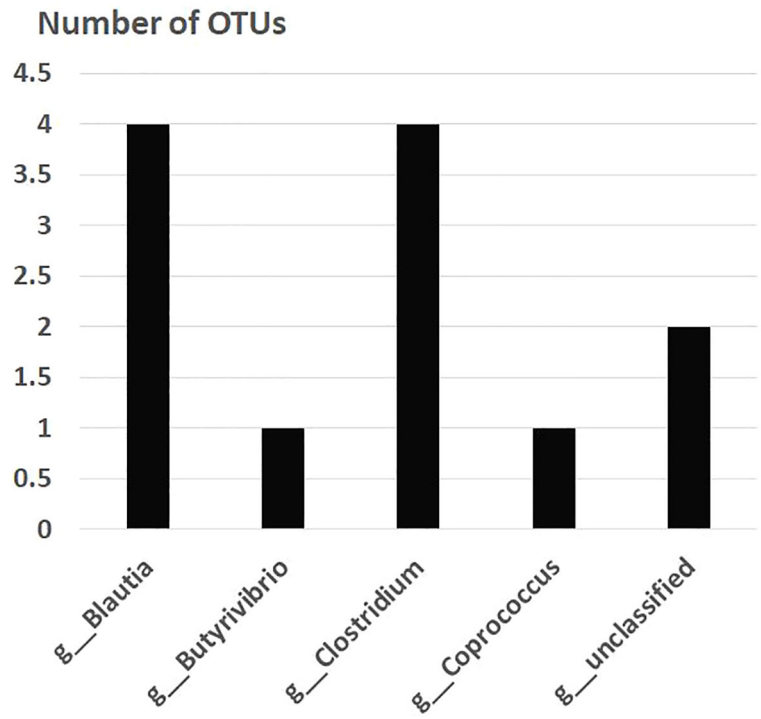

FIGURE 2
Overlapping gut bacterial diversities of family *Lachnospiraceae* in non-FMF and FMF men after the placebo administration (number of OTUs). $P < 0001$. FMF, Familial Mediterranean fever; OTUs, operational taxonomic units; g, genus.

diversities in non-FMF and FMF men after the placebo administration," it suggests that there were specific diversities of bacteria within the *Clostridiales* order that changed after the placebo course in healthy individuals but not in patients.

Resistance to placebo gut bacterial diversities in non-FMF and FMF men (number of OTUs)

The investigations found that FMF patients did not have unaffected bacterial genera after the placebo course. A limited number of changes were observed in the OTUs related to the genera of *Akkermansia*. Out of the 217 OTUs belonging to the genus *Akkermansia* (phylum: *Verrucomicrobia*), only 22 changed after the placebo administration (Table 6). Notably, at the species level, no changes were observed in OTUs related to *Akkermansia muciniphila* with all 108 OTUs remaining unaffected after the placebo course.

Discussion

Approximately 1,500 bacterial species, spanning over 50 different phyla within the intestinal microbiota, play a crucial role in maintaining normal human physiology and health (Conz et al., 2023). The colonic microbiota, boasting the greatest diversity, harbors up to 100 trillion bacteria. In the symbiotic relationship between bacteria and the host, gut bacteria collaborate with the host to ensure the well-being of the nervous system as well (Morais et al., 2021). The human gut microbiome exhibits gender-specific characteristics (Pepoyan et al., 2021; Bardhan and Yang, 2023) and potential interindividual variations (Chen et al., 2022; Afzaal et al., 2022; Wan et al., 2023). The link between the intestinal and systemic immune systems is primarily influenced by the expansion of the immune response through lymphatic and blood circulation (Zheng et al., 2020; Campbell et al., 2023; Li et al., 2022).

Currently, a wealth of data supports the notion that disruptions in the diversity of human gut bacteria, particularly a low level of bacterial diversity within the genus *Faecalibacterium*, can lead to undesirable consequences, such as inflammatory processes (Martín et al., 2023). In instances of inflammatory or metabolic diseases, a reduction in bacterial diversities is also noted in *Blautia* (Liu et al., 2021) and *Clostridium* (Guo et al., 2020). This may explain the extensive discussions surrounding the potential use of these bacteria as next-generation probiotics or living biotherapeutics (Martín et al., 2023; Guo et al., 2020).

Conversely, in the context of metabolic diseases, there is a growing focus on mucin-degrading species of *A. muciniphila* from the *Verrucomicrobia* phylum. This species has recently garnered significant attention and is widely discussed as a potential candidate for next-generation probiotics (Jian et al., 2023).

Preliminary evidence suggests the potential clinical utility of probiotics for FMF. Specifically, studies have demonstrated that a formulation containing eight bacterial strains, known as the De Simone Formulation and marketed as Vivomixx® in Europe and

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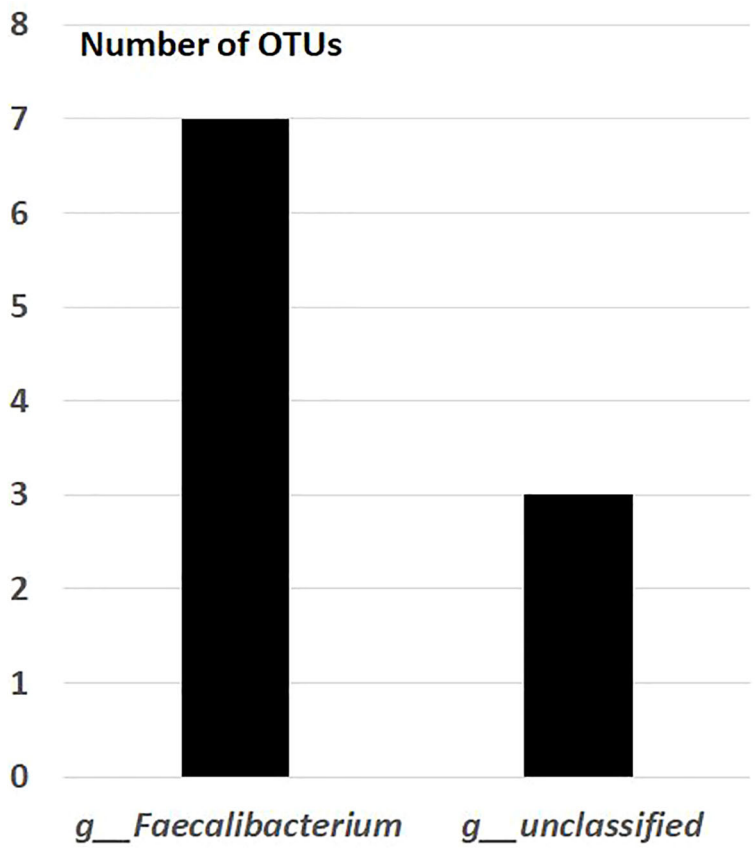

FIGURE 3
Overlapping gut bacterial diversities of family *Ruminococcaceae* in non-FMF and FMF men after the placebo administration (number of OTUs). $P < 0001$. FMF, Familial Mediterranean fever; OTUs, operational taxonomic units; g, genus; f, family.

Visbiome® in the US, may have beneficial effects when administered during the inter-critical period of FMF. This intervention shows promise in improving symptoms, particularly in a subgroup of FMF patients characterized by more severe disease and partial resistance to colchicine (Di Ciaula et al., 2020).

Additionally, our prior investigations evidence the impact of probiotic Narine (*Lactobacillus acidophilus* INMIA 9602 Er-2 strain 317/402) on the disease manifestation. Specifically, it has been shown that intake of *Lactobacillus acidophilus* INMIA 9602 Er-2 strain 317/402 has positive effects, including the normalization of serum C-reactive protein levels in FMF patients during remission (Pepoyan A. et al., 2015; Pepoyan et al., 2017; Balayan et al., 2015).

In addition to analyzing blood parameters, our previous studies also delved into the impact of the probiotic Narine on the composition of specific members of the intestinal microbiota in patients with FMF (Pepoyan et al., 2018a).

TABLE 6 Number of OTUs of resistant to placebo bacterial diversities.

Bacterial diversities	FMF people, N=15		Non-FMF people, N=15	
	Before taking placebo	After taking placebo	Before taking placebo	After taking placebo
Akkermansia (phylum *Verrucomicrobia*)	217	195 (89.86) ($P < 0.05$)	237	235 (99.12) ($P < 0.05$)
A. muciniphila	108	108 (100) ($P < 0.05$)	107	105 (95.33) ($P < 0.05$)
Enterobacteriaceae	1,228	686 (55.86)	0	0

*The impact of the placebo on 18,725 bacterial OTUs was evaluated; $P < 0.05$.
In parentheses- OTUs percentages.
FMF, familial Mediterranean fever.
OTUs, operational taxonomic units.
P, comparison of data (before and after placebo administration for the group).

In the context of FMF disease (Pepoyan et al., 2017; Balayan et al., 2015; Manzano et al., 2023; Touitou and Pepoyan, 2008; Lancieri et al., 2023; Pepoyan et al., 2019b), placebos are employed to evaluate the impacts of probiotics [microorganisms that confer beneficial effects on humans (Pepoyan et al., 2023; García-Santos et al., 2023; Harutyunyan et al., 2022), animals (Ataya et al., 2023; Balayan et al., 2019; Rodriguez et al., 2017; Wang et al., 2023; Mirzabekyan et al., 2023; Šefcová et al., 2023), and plant host metabolism (Rahman et al., 2018; Pepoyan and Chikindas, 2020; Mockevičiūtė et al., 2023)], along with their metabolites, as well as medications in general (Ben-Zvi et al., 2017; Haviv and Hashkes, 2016; Hashkes and Huang, 2015; Hashkes et al., 2014). While placebos have long served as inert controls in clinical trials (Gupta and Verma, 2013; Finniss et al., 2010; Louhiala and Puustinen, 2017), it is essential to recognize that placebo effects are psychobiological phenomena (Pogany, 2017; Hashmi, 2018; Liu, 2022; Schaefer et al., 2023; Shafir et al., 2023) capable of producing effects similar to certain drugs, even when patients are not knowingly given placebos (Bräscher et al., 2022). The term "placebo" originates from the Latin word "placere" (Schaefer et al., 2023; Yetman et al., 2021), meaning "to please" (Dreber et al., 2023). Surprisingly, approximately 40% of medications exhibit placebo effects (Sonawalla and Rosenbaum, 2002; Fässler et al., 2010; Pardo-Cabello et al., 2022; Moerbeek, 2023).

A "pure" placebo is typically represented by empty capsules (Welzel et al., 2021; Tsaturyan et al., 2023; Franc et al., 2022; Moerbeek, 2023) or inert substances like starch, dextromaltose, lactose, talc, mentholated water, and saline (Mitsikostas et al., 2020). The diversity of placebo effects is attributed to various biological mechanisms, influenced by the evolutionary development of the body's unique defense mechanisms (Benedetti, 2014; Buergler et al., 2023; Seneviratne et al., 2022; Pronovost-Morgan et al., 2023). Despite the literature data on placebo-dependent studies on the gut microbiota of FMF patients, in these studies, the placebo effect is discussed in part, depending on the nature of the problems presented in the articles. Perhaps, it was these incomplete discussions that pointed to the need for a full discussion of placebo effects on the gut microbiota of FMF patients. This study revealed distinct effects of a placebo on the bacterial diversities of the gut microbiota in both healthy and FMF-afflicted men, with a more pronounced impact observed in those with FMF.

Analysis of hybridization scores indicated that in non-FMF male subjects, the placebo induced quantitative changes in the altered main bacterial diversities, a phenomenon not observed in FMF male subjects.

Overlapping gut bacterial diversities in non-FMF and FMF men after the placebo administration

Considering the changed bacterial diversities observed in both healthy and diseased volunteers across studies, the overlapping gut bacterial variations should be carefully considered in placebo-dependent FMF-gut microbiota studies. Dysbiosis and inflammation in the gut have been associated with various mental illnesses, including prevalent conditions like anxiety and depression (Clapp et al., 2017). Moreover, the impact of gut bacteria on anxiety and depression levels appears to be influenced by gender (Pepoyan et al., 2021). It is conceivable that the overlapping gut bacterial diversities observed in non-FMF and FMF men after placebo administration represent key bacterial varieties with potential beneficial effects on anxiety and depression levels in both groups.

During the investigation, interviews were conducted to assess the anxiety and depression levels of the participants (Pepoyan et al., 2021). Even simple, non-test interviews indicated that after the placebo administration, both healthy individuals using the "pills" as an immunostimulant and patients felt more resilient to various infections and perceived themselves as healthier than before taking the "pills." This observation was supported by the placebo's effect on the psychoemotional status of men, potentially influenced by corresponding changes in intestinal bacteria.

The present research underscores the sensitivity of several species within the *Faecalibacterium, Blautia*, and *Clostridium* genera to the placebo effect. The significance of F*aecalibacterium* (Martín et al., 2023), *Blautia* (Liu et al., 2021), and *Clostridium* spp (Guo et al., 2020). in inflammatory/metabolic diseases is well-established. The observation that *Faecalibacterium*, *Blautia*, and *Clostridium* are influenced by the placebo effect could have noteworthy implications for clinical studies, particularly within the field of microbiome research. Clinical trials involving interventions that impact these bacteria must carefully consider the placebo effect, especially when evaluating the effectiveness of treatments targeting specific microbiota for diseases. It is crucial to understand how the placebo, including the type of capsule used (e.g., empty gelatin capsule), may affect these bacteria to accurately assess treatment outcomes. These findings are also significant for discussions regarding the potential use of *Faecalibacterium*, *Blautia*, and *Clostridium* spp. as probiotics.

These positive changes due to the placebo effect likely indicate that despite claims that it is ethically wrong to deceive people with placebos, it is still possible to prescribe placebos in extreme circumstances (for example, drug shortages).

Differences in gut bacterial diversities in non-FMF men after the placebo administration

Care should be exercised in interpreting the changes in bacterial diversities that occurred after the placebo course, especially when comparing healthy individuals and patients. In the presented study, it has been shown that there were bacterial diversities that changed after the placebo course in healthy individuals but not in patients.

This observation warrants further investigation. It is possible that the dietary habits of individuals with FMF may also influence the placebo effect (Mansueto et al., 2022).

Differences in gut bacterial diversities in FMF men after the placebo administration

As mentioned, the association between genetics and gut microbiota was recognized in FMF patients (Tsaturyan et al.,

2023; Pepoyan et al., 2018a). Following a placebo course in FMF patients, substantial changes were observed compared to healthy individuals. While some of these changes may be influenced by factors present in both healthy individuals and FMF patients, it was evident that these alterations should be duly considered in the design and interpretation of future clinical trials focused on gut microbiota in FMF patients.

Bacterial diversities that did not undergo changes after placebo

The investigations have uncovered that no bacterial genera were left unaffected after the placebo course in FMF patients. Conversely, there were no placebo-induced altered bacterial differences observed in *Enterobacteriaceae* diversities for non-FMF men. The *Enterobacteriaceae* spp. encompasses both pathogenic and commensal bacterial diversities, including commensal *Escherichia coli* (Tsaturyan et al., 2022; Pepoyan et al., 2020b). Prior research has highlighted that the prevalence of dominant commensal *E. coli* in the gut can vary depending on the health status of an individual (Shahinyan et al., 2003; Stepanyan et al., 2007; Mirzoyan et al., 2006; Pepoyan et al., 2014). In a study on *E. coli* isolates in colorectal cancer patients, Tang and colleagues concluded that "diseased" isolates suppressed the growth of healthy isolates under nutrient-limited culture conditions (Shandilya et al., 2021). This effect is possibly linked to altered gut-microbiota-mediated oxidative stress (Pepoyan et al., 2020b; Ni et al., 2022), a phenomenon also observed during FMF disease (Pepoyan et al., 2017).

These studies once again underscore the existence of a gut-brain connection.

Limitations of the study

Considering the qualitative changes in the microflora found during our research and the limitations that could affect the results of the research, more global studies including a larger number of participants have been planned to be conducted. Although the use of DNA-microarray-based data for analyzing gut microbiota is a powerful tool, it comes with potential biases and limitations (e.g., detection limitations of low abundance species, reference database bias, and limited quantitative accuracy). To address these limitations, using sequencing and quantitative PCR methods in further research to assess more clearly the qualitative and quantitative composition of the microflora is planned.

Conclusion

This study addresses the escalating demand for placebo-controlled trials by synthesizing knowledge on their impact on human gut microbiota, particularly in FMF patients. Beyond existing data on FMF patients, the focus is on identifying bacterial diversity unaffected by placebos for reliable use in clinical trials.

Noteworthy findings reveal that gut bacteria in healthy and FMF patients differ in their response to placebos. In healthy individuals, placebo minimally influences bacterial diversities, altering only 140 of 18,725 examined bacterial OTUs. Despite this limited change, all bacterial phyla are affected, excluding *Enterobacteriaceae* spp., which may be of value for studies involving healthy subjects.

Conversely, placebos affect all gut bacteria phyla in FMF patients, extending to nearly all bacterial genera. *Akkermansia* from the phylum *Verrucomicrobia* shows relative resistance, with only 22 out of 217 OTUs affected. *Faecalibacterium, Blautia*, and *Clostridium* genera exhibit susceptibility to placebo in both FMF and non-FMF men, showcasing distinct diversities altered after placebo administration.

Importantly, the study reveals that placebo-induced quantitative changes in bacterial diversities in non-FMF men differ from FMF male subjects, as indicated by hybridization scores. This study, critical for placebo-controlled trial design, not only lays the groundwork for exploring the gut-brain axis but also informs discussions on probiotic therapies involving *Faecalibacterium* spp., *Blautia* spp., and *Clostridium* spp.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GEO Series accession number GSE111835.

Ethics statement

The study was approved by the Ethics Committee at the Higher Education and Science Committee of Armenia (10-15-21AG; 21/10/2021).

Author contributions

EP: Formal analysis, Investigation, Writing – review & editing. FM: Writing – review & editing. AM: Investigation, Validation, Writing – review & editing. AG: Writing – review & editing. LS: Writing – review & editing. HG: Writing – review & editing. LG: Formal analysis, Writing – review & editing. MM: Writing – review & editing. MB: Investigation, Writing – review & editing. NH: Investigation, Writing – review & editing. SM: Investigation, Writing – review & editing. VT: Formal analysis, Supervision, Writing – review & editing. TT: Methodology, Resources, Software, Writing – review & editing. AP: Conceptualization, Data curation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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