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Dietary Trials and Gut Candidate Phyla Radiation Bacteria: The Effect of Placebo on the Prevalence of *Saccharibacteria* in Healthy Armenian Women and Women with Familial Mediterranean Fever

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ABSTRACT

"Candidate Phyla Radiation" (CPR) bacteria, representing ~15 % of bacterial diversity and over 70 phyla, are extremely small bacteria that primarily survive in parasitic or symbiotic forms. CPR bacteria, including Candidatus *Brownbacteria*, Candidatus *Hugbacteria*, and Candidatus *Saccharibacteria* (formerly TM7), were first identified in humans in 2007. They are linked to the microbiota of healthy and diseased individuals, being present in the oral cavity, gastrointestinal, and reproductive tracts. CPR bacteria, such as *Saccharibacteria*, are associated with dysbiotic conditions like periodontitis and can act as pathogens and potential protectors against inflammatory damage caused by host-associated bacteria.This study aimed to assess the effect of a placebo on gut *Saccharibacteria* in healthy Armenian women and those with Familial Mediterranean Fever (FMF) disease, a condition with high prevalence in Armenia and often associated with oral microbiota disturbances. Stool samples were analyzed using a culture-independent, high-density DNA microarray method, and statistical analyses were performed with Multibase 2015 Excel Add-in program (NumericalDynamics, Tokyo, Japan). Results indicate that *Saccharibacteria* respond variably to placebo depending on health status, with some showing significant quantitative or qualitative changes while others remained unchanged. In conclusion, this study confirms the presence of CPR bacteria in the gut microbiota of both healthy women and those with FMF. The distinct responses of intestinal CPR bacteria to placebo highlight the importance of placebo-controlled trials in microbiota research. Furthermore, the findings emphasize the potential role of *Saccharibacteria* in gut-brain processes and their implications in health and disease.

Introduction

Candidate Phyla Radiation bacteria, which make up about 15 % of total bacterial diversity and over 70 phyla, represent a recently discovered group. The latter show minimal functional variability and are classified as obligate fermenters. (Danczak, et al., 2017). CPR bacteria are characterized by their extremely small cell sizes,

simplified genomes, and limited metabolic capabilities. They are hypothesized to survive in parasitic or symbiotic forms (Ji, et al., 2022). Examples of CPR bacteria include Candidatus Brownbacteria and Candidatus *Hugbacteria* (Danczak, et al., 2017). The presence of nirK in four *Parcubacteria* genomes, which encodes nitrite reductase, indicates the potential role of these microorganisms in the denitrification process (Danczak, et al., 2017). Some representatives found in various underground niches show potential for carbon processing, which allows them to degrade a wider range of carbon substrates compared to other CPR phyla (Danczak, et al., 2017). Certain proteins unique to CPR bacteria regulate certain cell-to-cell interactions, which are crucial for their episymbiotic lifestyle (Méheust, et al., 2019). A detailed analysis of the CPR-C4 protein has enabled the identification of a non-canonical cysteine-histidine-leucine (carbonyl) catalytic triad in this protein. Structural and functional similarities between CPR-C4 and human vaso-hemoglobins suggest evolutionary relationships (Cornish, et al., 2022).

CRISPR targeting in the *Microgenomates superphylum* has identified three phages and prophages with complete genomes. Interestingly, one of these phages encodes a Cas4-like protein. *Roizmanbacteria*, which possess CRISPR loci, include a fragmented CasY gene (fCasY). These studies on CPR bacteria expand our understanding of CasY diversity and, more broadly, provide insights into the CRISPR-Cas systems and the phages associated with CPR bacteria (Chen, et al., 2019). CPR bacteria were first identified in humans in 2007 as part of the oral microbiota (Liu, et al., 2024; Allison, et al., 2024; Hu, et al., 2024; Rajasekaran, et al., 2024; van der Ploeg, et al., 2024; Baker, et al., 2017; DeDe Kwun Wai et al., 2024). In both healthy and diseased states, the human oral microbiota is associated with CPR bacteria such as *Gracilibacteria*, *Absconditabacteria*, and *Saccharibacteria* (formerly known as TM7). Subsequently, CPR bacteria have also been described in the human gastrointestinal and reproductive tracts, indicating that these bacteria constitute a minor part of the human microbiota. The prevalence of CPR bacteria is also noted in pathological states, under dysbiotic conditions. (Naud, et al., 2022, Jaffe, et al., 2024, Zhu, H., et al., 2024). Candidatus *Saccharibacteria* are regarded as the most widespread bacteria found in the oral microbiota. (Bor, et al., 2019). They have "host" bacteria and, in fact, act as epibionts residing on the surface of the latter (Chipashvili, et al., 2021). The presence of *Saccharibacteria* is primarily associated with periodontitis, suggesting that these bacteria may act as pathogens. However, Chipashvili and co-authors also conclude that the species isolated from individuals with periodontitis may protect mammals from inflammatory damage caused by host-associated bacteria (Chipashvili, et al., 2021).

In dietary and clinical trials, the effects of nutrients or drugs on the composition and prevalence of gut microbiota and its individual members are frequently compared to the effects of a placebo, an "inert" substance (Munnangi, et al., 2023; Khneizer, et al., 2022; Gupta, et al., 2013; van de Put, et al., 2024; Staudacher, et al., 2017; Pepoyan, 2024a; Lieb, et al., 2020; Jung, et al., 2023; Pepoyan E., et al., 2024). Probiotics have become increasingly used in recent years to regulate dysbiotic conditions in organisms humans (Latif, et al., 2023; Sulaimany, et al., 2024; Tsaturyan, et al., 2022; Jan, et al., 2024; Jangi, et al., 2024; Mirzabekyan, et al., 2023; Pepoyan, et al., 2018a; Pepoyan, et al., 2009), animals (Mirzabekyan, et al., 2023; Pepoyan, et al., 2020a, b, c, d; Manvelyan, et al., 2023; Pepoyan, et al., 2023, 2024) and plants (Harutyunyan, et al., 2022). In nutritional and clinical trials involving probiotics, comparisons are also made against placebo (Kazemi, et al., 2019; Lazou-Ahrén, et al., 2024; Jukic Peladic, et al., 2021; Colletti, et al., 2023; Virk, et al., 2024).

Considering that the composition of the normal human intestinal microbiota is influenced by factors such as genetics (Goodrich, et al., 2014; Goodrich, et al., 2017; Tsaturyan, et al., 2023; Hou, et al., 2022), diet (Rinninella, et al., 2023; Martínez, et al., 2024; Zhang, 2022; González Olmo, et al., 2021), sex (Kim, et al., 2020; Fransen, et al., 2017; Tsai, et al., 2022; Pepoyan, et al., 2021; Galstyan, et al., 2008), healthy or diseased states (Belli, et al., 2023; Yeoh, et al., 2021; Borzan, et al., 2023; Hou, et al., 2022; Van Hul, et al., 2024; Afzaal, et al., 2022; Kho, et al., 2018), environmental factors (Pepoyan, et al., 2023; De Filippis, et al., 2024; Singh, et al., 2022; Jernfors, et al., 2024; Chiu, et al., 2020), and given that Familial Mediterranean Fever (FMF) is relatively common in Armenia (Balayan, et al., 2015; Pepoyan, et al., 2015a; Pepoyan, et al., 2019a, b) and that individuals with FMF often experience oral cavity-related issues (Sogur, et al., 2013; Abouzaid, et al., 2022), the aim of this preliminary study was to characterize the effect of placebo on the prevalence of *Saccharibacteria* (TM7) in the intestinal microbiota of healthy and FMF-affected Armenian women.

Materials and methods

Our previous works describe the approaches and methods (Tsaturyan, et al., 2023; Pepoyan, et al., 2015b) for conducting the present research, and the datasets are available www.ncbi.nlm.nih.gov ((GSE111835 study at: www.ncbi.nlm.nih.gov) accessed on March 16, 2008, last modified on July 16, 2018)). This method also made it possible to study

differences in the relative abundance of bacterial taxa based on differences in hybridization intensity.

The bacterial analysis of stool samples was performed using the third-generation, culture-independent, high-density DNA microarray method described by Kellogg and co-authors. As a placebo, high-quality empty gelatin capsules commonly used in pharmaceutical preparations were utilized (Kellogg, et al., 2013; Pepoyan, et al., 2018b). PhyloChip™ DNA microarray, which allows the evaluation of more than 50,000 individual bacterial taxa, also made it possible to study differences in the relative abundance of bacterial taxa based on differences in hybridization intensity. Seven operational taxonomic units (OTUs) of Candidatus *Saccharibacteria spp.* in the human intestinal microbiota were analyzed using the Multibase 2015 Excel Add-in program (NumericalDynamics, Tokyo, Japan).

Results and discussions

The hybridization scores for gut Candidatus *Saccharibacteria* species (formerly TM7) were analyzed to evaluate their response to placebo administration in healthy and FMF-affected women (Table). The data indicate distinct response patterns between the two groups, highlighting potential differences in microbiota dynamics based on health status.

In FMF-affected women, no statistically significant changes in hybridization scores were observed for any strains (P>0.05) following placebo administration. Strains Str.1, Str.3, and Str.4 exhibited minimal fluctuations in scores, with values before and after placebo administration remaining comparable (e.g., Str.1: 6208±1320 vs. 6034±1297, P=0.83). Strain Str.6 showed a notable increase in intensity (3334±936 before vs. 4273±691 after); however, this change was not statistically significant (P=0.16).

In healthy women, in contrast to the FMF group, several strains demonstrated changes in hybridization scores following placebo administration: strain Str.5 showed a significant reduction in intensity after placebo (558±330 vs. 358±194, P = 0.04), indicating a placebo response, Strain Str.6 also exhibited a significant decrease in scores post-placebo administration (4609 ±1761 vs. 3346 ±1047, P=0.02). Strain Str.7 approached statistical significance (P=0.05), with a decrease in hybridization scores from 3151±1581 to 2352±784. Other strains, including Str.1, Str.3, and Str.4, showed no significant differences before and after placebo administration (P>0.05).

In summary, the stability of scores across all strains in FMF-affected women contrasts with the changes observed in healthy women, particularly for strains Str.5, Str.6, and Str.7. This divergence suggests that the gut microbiota response to placebo may depend on underlying health conditions, with FMF potentially influencing the resilience or responsiveness of Candidatus Saccharibacteria species.

At the same time, a study of the quantitative/qualitative prevalence of Candidatus Saccharibacteria in the intestinal microbiota of healthy women and women with FMF disease showed that there were no significant quantitative differences between these bacteria in the intestinal microbiota of healthy and FMF patients (P > 0.05; P values not shown in Table).

Table. The intensity of hybridization scores of gut Candidatus Saccharibacteria (former TM7) spp. before/after placebo administration in healthy and FMF-affected women*

Strains	Intensity					
	FMF women			Healthy women		
	Before	After	P before/after	Before the placebo	After the placebo	P before/after
Str.1	6208±1320	6034±1297	0.83	5254±1203	5266±992	0.29
Str.2	910±323	867±205	0.64	846±587	539±318	0.06
Str.3	202±118	402±310	0.28	1023±1499	403±633	0.12
Str.4	6178±2494	5388±2567	0.58	4244±1805	4576±1692	0.45
Str.5	422±165	438±205	0.91	558±330	358±194	0.04
Str.6	3334±936	4273±691	0.16	4609±1761	3346±1047	0.02
Str 7	3552±1793.54	2960±830	0.47	3151±1581	2352±784	0.05

*Composed by the authors.

An intriguing finding is that Saccharibacteria exhibit no significant quantitative differences in the gut microbiota between healthy women and those with familial Mediterranean fever (FMF). As previously noted, oral diseases are prevalent among FMF patients. In light of these observations, it can be hypothesized that women with FMF may experience a shift in the oral-intestinal microbiota.

Additionally, it is noteworthy that intestinal Candidatus Saccharibacteria may exhibit variable responses to placebo, contingent upon the individual's health status. This variability could potentially be attributed to the unique characteristics of the gut-brain axis in healthy versus diseased individuals.

The current study reaffirms the existence of these features and provides evidence of the involvement of intestinal Saccharibacteria in gut-brain axes. The distinctive characteristics of this relationship, influenced by the manifestations of depression in female patients with familial Mediterranean fever, have been demonstrated in our previous studies (Pepoyan, et al., 2021). Based on the findings of this study, role of basibiont bacteria of Candidatus Saccharibacteria in the gut-brain axis is also suggested. However, these hypotheses necessitate further investigation through advanced molecular biological studies, which are planned for future research endeavors.

Conclusion

Currently, microbiological research is undergoing rapid development. The advancements have led to the discovery of CPR bacteria. These bacteria were first described as "ultramicrobacteria" with cell sizes smaller than 0.1 μm³ in 1981. CPR bacteria are predominantly found in the microbiomes of subsurface waters and are characterized by low biomass. Although CPR bacteria are relatively rare within the human microbiota and are predominantly associated with the oral microbiota, they are also present, albeit in low concentrations, as constituents of the normal intestinal microbiota. The presence of Saccharibacteria in individuals with conditions such as familial Mediterranean fever may highlight their role in mediating specific health responses through the gut-brain axis. This group of bacteria, while still under investigation, could offer insights into how gut microbiota composition influences health conditions with a neurological component. This study highlights the potential role of Candidatus Saccharibacteria in the gut microbiota, particularly in the context of FMF. Although their quantitative prevalence did not differ significantly between healthy women and those with FMF, notable differences in responsiveness to placebo administration were observed. In healthy women, certain strains exhibited significant changes, whereas FMF patients showed no significant alterations, suggesting that FMF may influence microbiota stability and responsiveness.

The findings support the potential involvement of Candidatus Saccharibacteria in the gut-brain axis, with their dynamics influenced by host health status. Additionally, the potential shift in oral-intestinal microbiota in FMF patients highlights the interconnectedness of microbiota across anatomical sites.

These results emphasize the need for further molecular studies to confirm the functional role of Candidatus Saccharibacteria in human gut and gut-brain health and their implications for conditions like FMF.

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Declarations of interest

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