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The gut microbial composition is different in chronic fatigue syndrome than in healthy controls

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The pathogenesis of Chronic Fatigue Syndrome (CFS) is yet unknown. This study aimed to assess the gut microbial composition in CFS patients versus in healthy controls (HCs). The composition of fecal bacteria was examined in twenty-five CFS patients and sixteen HCs using Illumina sequencing of 16 S rRNA gene amplicons targeting the V3-V4 bacterial gene regions. 143 (46%) of the microbial genera were found only in the CFS. In addition, the gut microbial composition in the CFS patients contained a much higher proportion of the 10 most commonly found bacteria compared to the HCs group. A significantly lower observed number of operational taxonomic units (OTUs) was noted in CFS compared to HCs ($p = 0.045$). Significant between-group differences in the gut microbial composition in CFS compared to HCs were noted. The three most discriminating Amplicon Sequencing Variants (ASVs): ASV 191, ASV 44, and ASV 75, were identified as significantly more abundant in the healthy control group compared to the patient group. In addition, the Neural Network (multilayer perceptron) was able to discriminate gut microbial composition from CFS versus HCs with excellent performance (AUC = 0.935). The gut microbial composition is different in CFS patients compared to HCs. Further studies should assess the pathophysiological consequences of these differences as well as the effectiveness of therapies aimed at modifying the gut microbial composition in CFS patients.

Keywords Gut microbial composition, Enteric bacteria, Chronic fatigue syndrome, Myalgic encephalomyelitis, Cognitive function, Brain fog

Abbreviations

AD	Alzheimer's disease
ASV	Amplicon Sequencing Variants
AUC	Area under the ROC Curve
BDNF	brain-derived neurotrophic factor
BMI	body mass index
CD	Crohn's disease
CFS	Chronic Fatigue Syndrome
dbRDA	Distance-based ReDundancy Analysis
GBA	gut-brain axis
GM	gut microbiome
HCs	healthy controls
HCC	hepatocellular carcinoma
IBD	inflammatory bowel disease
ME	Myalgic Encephalomyelitis

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MCI	mild cognitive impairment
MDS1	first multi-dimensional scaling
ML	machine learning models
NAFLD	non-alcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
PEM	post-exertional malaise
rRNA	ribosomal ribonucleic acid
sPLS	DA-the Partial Least Squares Regression-Discriminant Analysis
SVM	Support vector machines
OTU	operational taxonomic units
TMT A and B	Trail Making Test A and B
UC	ulcerative colitis

Chronic Fatigue Syndrome (CFS), also known as Myalgic Encephalomyelitis (ME), is a multisystemic disease that significantly impairs daily activity and reduces the quality of life[1,2]. CFS symptoms include debilitating fatigue, post-exertional malaise (PEM), unrefreshing sleep, cognitive dysfunction (including memory and concentration difficulties), and symptoms of dysautonomia[2]. The exact pathogenesis of CFS is still unknown. Potential causes include infectious and immunological factors, genetic predisposition, and mitochondrial impairment[3]. Gastrointestinal disturbances are commonly reported by CFS patients. It is possible that one of the CFS causative factors can be gut microbial composition alterations[4].

Gut dysbiosis has been implicated in the pathogenesis of many diseases[5–10]. However, only a few studies on the composition of the gut microbial composition have been conducted in patients with CFS, and its correlation with the progression of CFS has not been confirmed[1,3].

It is hypothesized that enteric dysbiosis leads to the formation of abnormal metabolites and disrupts signaling via the gut-brain axis (GBA), which may reach the central nervous system and induce CFS symptoms such as cognitive and memory impairment, fatigue, or 'brain fog'[11,12]. Elucidating the involvement of gut dysbiosis in CFS etiology may improve diagnostics, through the discovery of biomarkers, and enable causative treatment via gut microbial composition modification[11].

The main aim of the current study was to compare the gut microbial composition of CFS patients to healthy controls (HCs). We hypothesized that alpha-diversity (within-sample diversity) and beta-diversity (between-sample diversity) of the enteric bacteria would significantly differ between CFS patients and HCs. To assess the gut microbial composition qualitatively and quantitatively, we used a novel network analysis and machine learning methods. To our knowledge, this is the first study to measure the association between the enteric bacteria composition and objectively measured cognitive function using a validated test (TrailMaking Test A and B, TMT A and B).

Results

Characteristics of the study population

Twenty-five CFS patients (20 females) and sixteen HCs (10 females) were examined. No statistically significant differences were observed in age, BMI, free-fat mass (FFM), and adipose tissue (Table 1). FFM was specifically compared between patients and controls because it may decrease due to prolonged inactivity, potentially serving as an indirect indicator of disease severity progression.

Relative abundance of gut microbial composition

The results of gut microbial composition analysis, described below, are also presented in Fig. 1 and Supplementary Figure S1: Fig. 1A shows the distribution of bacteria in the fecal bacteria of CFS patients and healthy control subjects at the phylum level, Fig. 1B at the genus level, while Figure S1A at the class level, Figure S1B at the order level, Figure S1C at the family level. We observed relative differences in the qualitative composition of the gut microbioal composition between CFS patients and healthy controls at each taxonomic level. At the phylum level, we observed higher *Bacteroidetes* and lower *Firmicutes* abundance in CFS. At the class level, we observed higher *Bacteroidia* abundance and lower *Clostridia*, *Negativicutes*, and *Actinobacteria* abundance in CFS patients. At

Parameter [units]	Group	Median	SE	*p*-value
Age [years of age]	CFS	36.0	1.7	0.72
	HCs	34.5	2.3	
BMI [kg/m^2]	CFS	24.4	0.9	0.84
	HCs	23.4	1.5	
FFM [kg]	CFS	51.5	1.8	0.64
	HCs	53.7	2.7	
Adipose tissue [%]	CFS	29.9	1.6	0.64
	HCs	25.5	2.10	
Visceral adipose tissue [level]	CFS	5.0	0.5	0.69
	HCs	4.5	0.8	

Table 1. CFS vs. HCs demographic and body composition comparison.

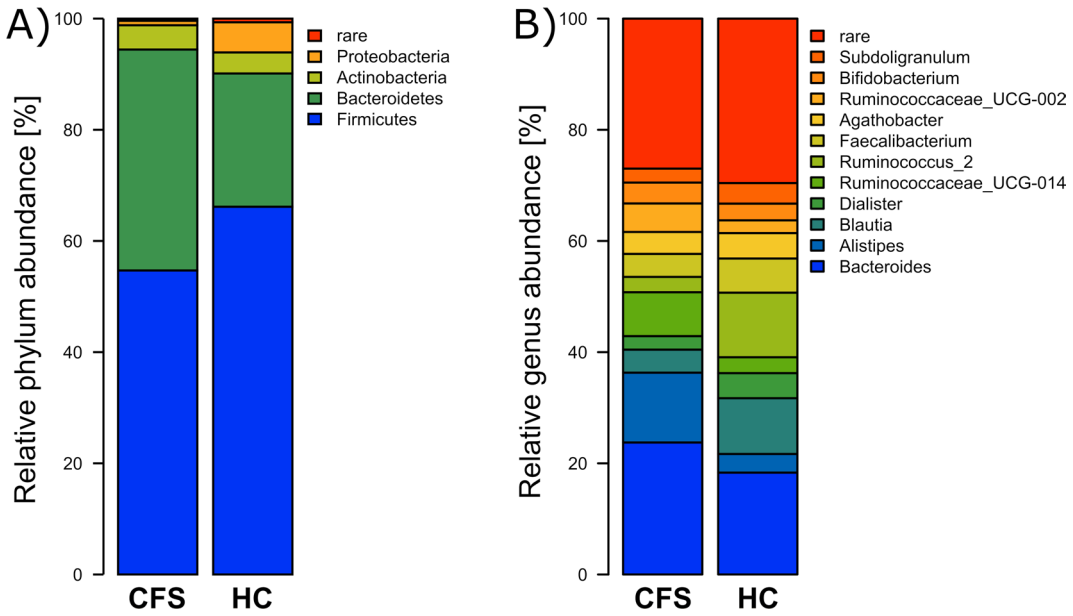

Fig. 1. Relative abundance of enteric bacteria in the phylum (**A**) and genus (**B**) level of individuals with CFS and healthy controls.

the order level, the abundance of *Bacteroidales* was higher, and *Selenomonadales* was lower in CFS. At the family level, the abundance of *Bacteroidaceae* was higher, and *Lachnospiraceae* and *Veillonellaceae* were lower in CFS. At the genus level, the abundance of *Bacteroides*, *Alistipes*, and *Ruminococcaceae* was higher in CFS patients than in healthy controls.

Alpha-diversity analysis

No significant differences in diversity (Shannon's H) and evenness (Shannon's E) were observed between the studied groups. The detailed results were presented in the Supplementary Table S1. A significantly lower richness (the total number of species in the unit of study), indicated by an observed number of operational taxonomic units (OTUs), was observed in CFS patients compared to HCs ($p = 0.045$), as shown in the Supplementary Figure S2.

Figure S3, available online, shows the number of common (middle of the graph) and different genera of bacterial species in the gut microbial composition in the analyzed groups. One hundred thirty-three (43%) genera were shared by both groups. Thirty-three (11%) genera were present in healthy controls only, while 143 (46%) were present in the CFS only. Supplementary Figure S4 shows differences in the prevalence of the 50 most common ASVs in the gut microbial composition of the studied groups. The CFS fecal bacteria was characterized by a lower abundance of the 10 most common ASVs compared to the HCs group.

Beta-diversity analysis

Beta-diversity analyses were performed on rarefied ASV abundance data (microbial taxa counts) as predictors. Taxa with near-zero variance were excluded. Both Distance-based ReDundancy Analysis (dbRDA) and PERMANOVA results showed significant between-group differences in beta-diversity of the gut microbial composition in CFS compared to HCs ($p = 0.046$ and $p = 0.044$, respectively). We used a sparse version of the Partial Least Squares Regression-Discriminant Analysis (sPLS-DA) to examine which ASVs best discriminated CFS from HCs. The gut microbial composition from the CFS was discriminated perfectly from HCs (AUC = 1) (Fig. 2A). The three most discriminating ASVs (genus) were: ASV 191 (*Oscillibacter*), ASV 44 (*Ruminococcus_2*), and ASV 75 (*Roseburia*), which were more abundant in HCs group (Fig. 2B).

Relationship of gut microbial composition with cognitive function

A graphical representation of interactions between microbial compositionand severity of cognitive symptoms measured by TMT A and B was carried out using dbRDA (Fig. 3). As could be seen, most of the data points indicating the gut microbial composition of particular subjects are not included in the dashed ellipses indicating 95% confidence, which suggests rather high between-subjects heterogeneity of gut microbial composition in both groups. The results indicate that both patients with CFS and healthy controls (HCs) who had worse TMT scores exhibited similar gut microbial compositions. PERMANOVA analysis revealed significant interactions between the gut microbial composition and cognitive function measures: specifically, TMT Part B scores (Fig. 3A) and the difference between TMT Part B and Part A scores (Fig. 3B), with p-values of 0.025 and 0.007, respectively. In the figures, triangles and circles represent beta-diversity results from distance-based redundancy analysis (dbRDA) using Bray-Curtis dissimilarity, with triangles for HCs and circles for CFS patients. The spatial arrangement of these markers is identical across panels since they are based on the same data. The difference

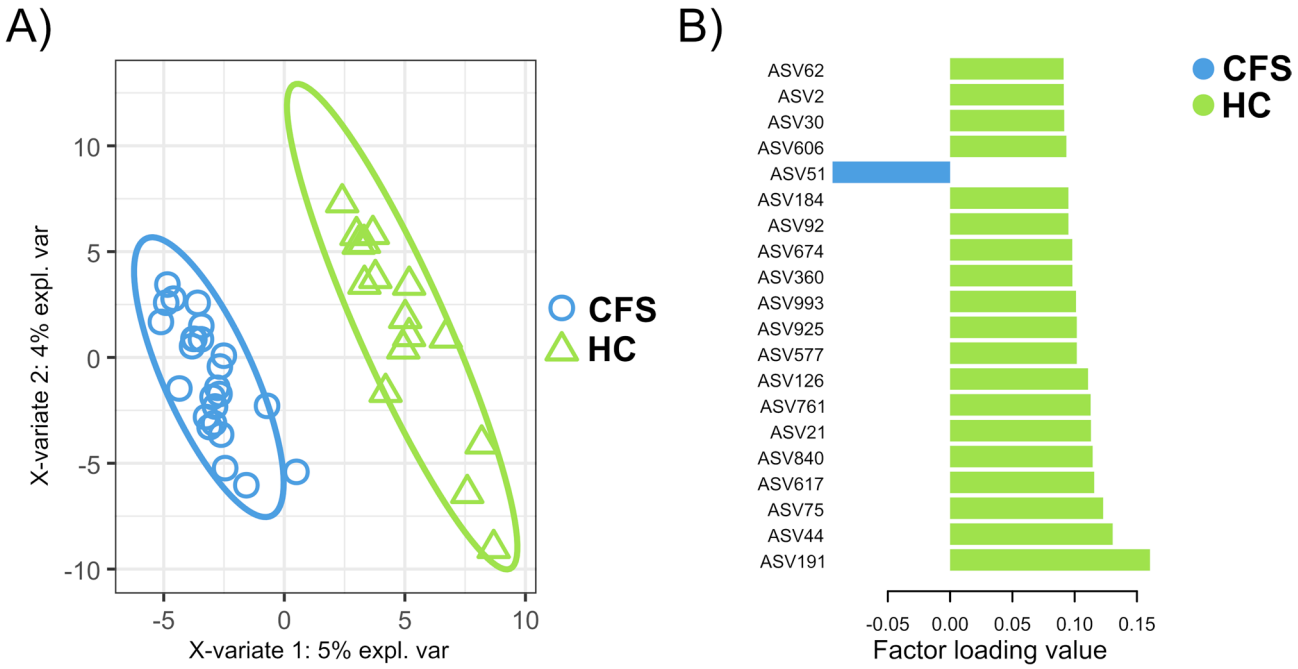

Fig. 2. Identification of a bacterial signature discriminating CFS and HCs with sPLS-DA. **A**) Data from CFS patients are shown in blue open circles, while data from HCs are shown in green open triangles. Dashed ellipses show 95% confidence, indicating a strong discrimination between CFS and HC. **B**) The length of the bars indicates the importance of each ASV in the signature (from bottom to top: decreasing importance), with colour indicating the phenotype group (blue for CFS and green for HCs) with maximum median abundance.

lies in the circle fill color: in panel A, the intensity of red corresponds to the TMT Part B score, while in panel B, it reflects the difference between Part B and Part A scores. A more intense red fill indicates worse cognitive performance in both cases.

Figure 4 shows the network tree heatmap for CFS (Fig. 4A) and HCs (Fig. 4B) groups. Node sizes and edge widths, and color intensity between them, are proportional to the relative abundance of the enteric bacteria in the particular parts of the tree of life (the greater the abundance, the more intensive the green color of an edge is). In addition, nodes denoting genera are filled with values of Spearman rho coefficient correlation between particular genera and the result of TMT B. Negative correlations are shown in blue, while positive ones are shown in red (Fig. 4). For simplicity, only coefficients with an absolute value of 0.4 are shown. In the CFS, a correlation of TMT B score was observed with 8 genera, and all of those relationships are negative (i.e., the higher the abundance, the lower the cognitive function test score is, indicating better cognitive function). In comparison, 29 correlations were noted between TMT B score and genera in the HCs; 9 of them were negative, while 20 correlations were positive. In both CFS and HCs, *Ruminococcaceae UCG*–014 is negatively related to the TMT B result, i.e., the higher the abundance, the better executive function is (rho=−0.44 and rho=−0.52, respectively). In contrast, the correlation between *Ruminococcaceae*_UCG-010 and TMT B score was positive in the control group and negative in CFS patients (rho = 0.63 and rho=−0.42, respectively). The rest of the genera that correlated negatively with TMT B score in the CFS had correlation levels lower than absolute 0.4 in healthy controls (*Christensenellaceae*_R-7_group (rho=−0.41), *Faecalibacterium* (rho=−0.49), *Prevotella*_9 (rho=−0.42), *Roseburia* (rho=−0.53), *Ruminiclostridium*_6 (rho=−0.41), and *Terrisporobacter* (rho=−0.58).

Comparison of network analysis results for a network based on ASV in the CFS compared to the HCs

Network analysis of the gut microbial composition at the Amplicon Sequence Variant level in the CFS compared to the HCs is presented in Fig. 5. In creating Fig. 5, a "union" layout was used so that the nodes were placed as optimally as possible, i.e., equally for both networks. In addition, individual nodes not connected to others were removed. Node sizes are scaled to the sum of the values for each ASV. Node colours represent specific clusters. The edges representing positive relationships between the values of individual ASVs (i.e., when the value of one ASV increases, the value of the other ASV also increases) are marked in green, and negative (when the value of one ASV increases, the value of the other decreases) are marked in red. Qualitative differences in the microbial composition network between the groups were observed and are presented in detail in Supplementary Table S2.

For example, there was a strong positive association between ASV20 (*Dorea*) and ASV2 (*Bacteroides*) in the CFS, while no such association existed in the HCs group. No strong negative relationships were found between individual ASVs in either group. The network created based on the HCs gut microbial composition was characterized by a significantly higher degree (an indicator of how many edges each node has, $p = 0.01$) and

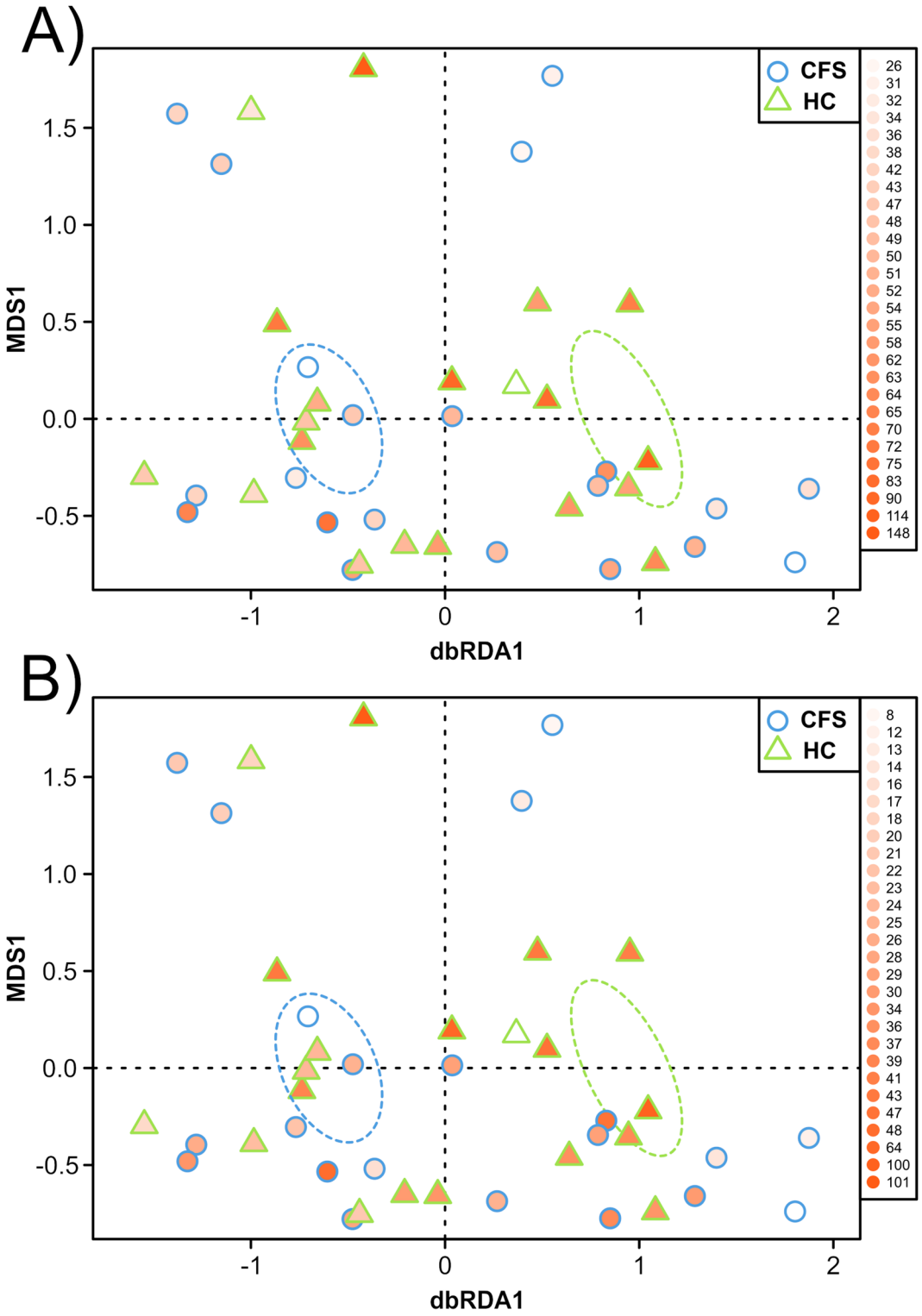

Fig. 3. Beta-diversity of gut microbial composition in the studied groups and interaction with results of Trail Making Test part B (TMT B (panel A) and the difference between Trail Making Test part B and A (TMT B-A (panel B)). Beta-diversity was assessed by distance-based redundancy analysis (dbRDA) on Bray-Curtis dissimilarity calculated on rarefied community data. Data from individual CFS patients is denoted as blue circles, while data from HCs is shown in green triangles. Dashed ellipses show 95% confidence. Shades of red inside circles and triangles denote the score of the cognitive function test: the higher the score, the darker the shade is (presented inside the box in the upper right corner). Differences across groups are established by constrained ordination at the first axis of the distance-based RDA (dbRDA1) ordination and the first multi-dimensional scaling (MDS1).

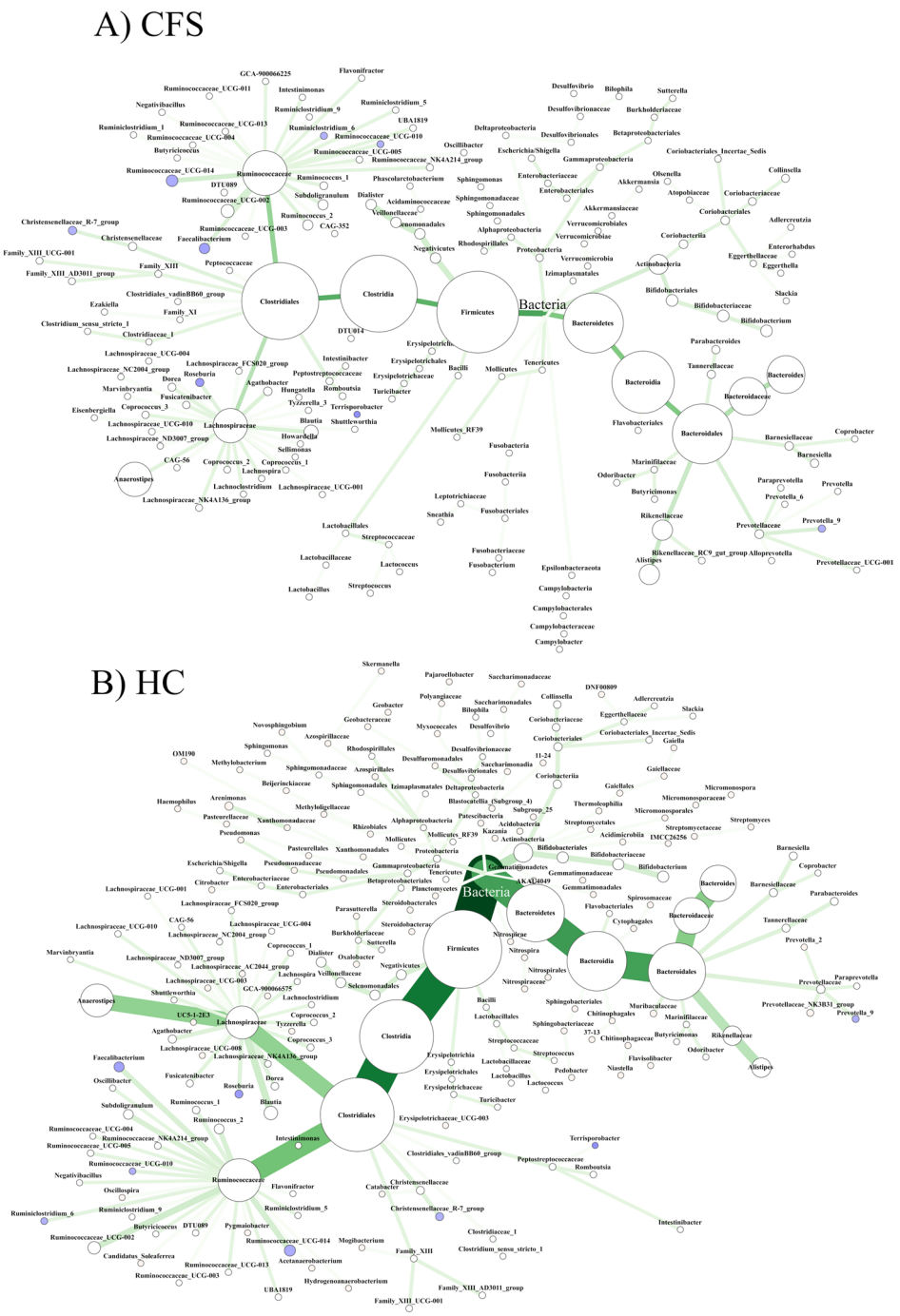

Fig. 4. Network tree heatmap showing correlation of Trail Making Test part B (TMT B) with mean abundance of genera in CFS (panel A) and HCs (panel B) groups. Nodes represent the abundance of enteric bacteria on the phylum, class, order, family, and genus levels. Edges connecting nodes represent the tree of life, and their width and intensity of green color are continuously related to the abundance of bacteria. The size of the dots next to the variable names is continuously related to the mean abundance. nodes denoting genera are filled with values of Spearman rho coefficient correlation between particular genera and the result of TMT B. Negative correlations are shown in blue, while positive ones are shown in red (Fig. 4). For simplicity, only coefficients with an absolute value of 0.4 are shown.

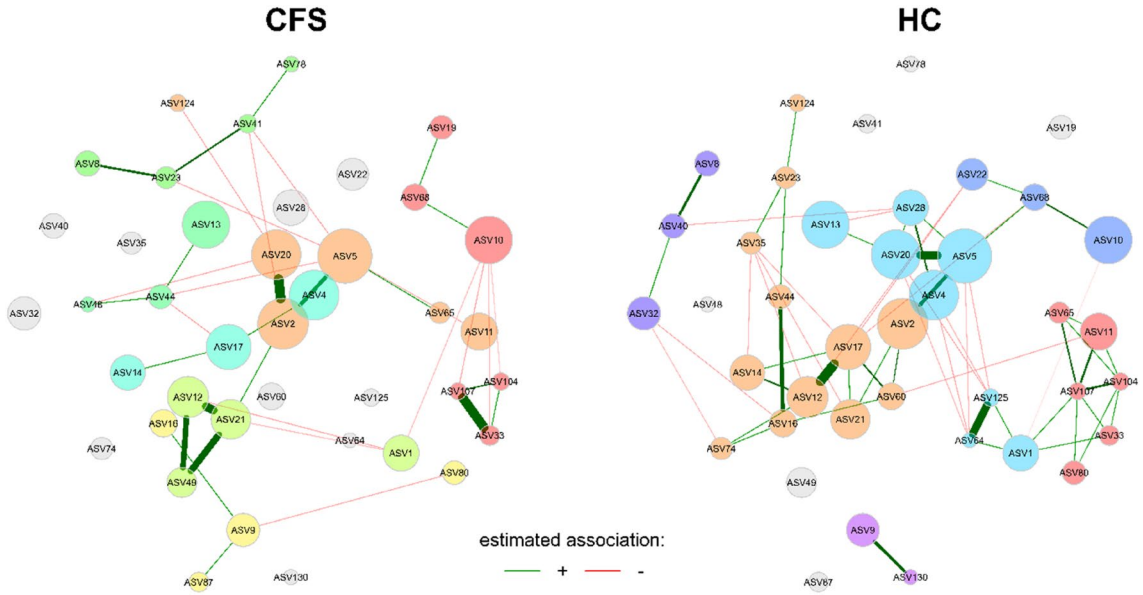

Fig. 5. Network analysis of the gut microbial composition at the amplicon sequence variant (ASV) level in the CFS compared to the HCs group.

Model	AUC	CA	F1	Precision	Recall	MCC
Neural Network	0.935	0.902	0.903	0.908	0.902	0.804
SVC	0.945	0.854	0.845	0.882	0.854	0.710
LR (Ridge)	0.825	0.805	0.805	0.805	0.805	0.590
LR (Lasso)	0.708	0.732	0.730	0.729	0.732	0.430
XGBoost RF	0.663	0.707	0.689	0.706	0.707	0.361
Catboost	0.722	0.707	0.689	0.706	0.707	0.361
XGBoost	0.740	0.683	0.681	0.680	0.683	0.327
AdaBoost	0.608	0.659	0.637	0.647	0.659	0.244
Random Forest Classifier	0.875	0.610	0.462	0.372	0.610	0.000

Table 2. Comparison of the performance of three classification models of CFS patients compared to the control group using machine learning methods. SVC-Support Vector Classifier, LR-Logistic Regression, XGBoost-eXtreme Gradient Boosting, RF-Random Forest, AdaBoost-Adaptive Boosting, AUC-Area under the ROC Curve, CA-Classification Accuracy, MCC- Matthews correlation coefficient.

eigenvector centrality (a measure of the influence of a node in a network, $p = 0.001$). No differences in global network properties were observed (p-values > 0.05).

Comparison of the effectiveness of machine learning models in the classification of patients with CFS compared to the HCs

The performance of nine machine learning classifiers was compared. The highest values of AUC (Area under the ROC Curve) were obtained by Neural Network (multilayer perceptron) and SVM. In contrast, XGBoost, Random Forest, and AdaBoost failed to obtain AUC values in the classification of patients with CFS compared to HCs, and did obtain an AUC value not considered 'acceptable' (all were lower than 0.7). The comparison of the performance of three classification models of CFS patients compared to the control group using machine learning methods is shown in Table 2. The top 3 ASVs with the best discrimination capability were ASV 3979, ASV 5362, and ASV 272 (as shown in Supplementary Figure S5 online).

Discussion

The current study constitutes an important subject about gut microbial composition in CFS. We observed significant differences in alpha-diversity with the major outcome that there was a lower number of operational taxonomic units (OTUs) noted in CFS patients' microbial composition. In beta diversity analysis, we designated three of the most discriminating enteric bacteria genera, which are partially consistent with previous research results. The current study was the first to assess the association between gut microbial composition and objectively measured brain fog severity using validated scales. We observed that higher levels of the following genera

were associated with better executive function in the CFS (*Christensenellaceae*_R-7_group, *Faecalibacterium*, *Prevotella*_9, *Roseburia*, *Ruminiclostridium*_6, and *Terrisporobacter*), while no such relationship was observed in the HCs.

In previous studies, researchers were using different methods, different patient cohorts, and different levels of molecular profiles, so it is challenging to integrate, compare, and interpret those data. Therefore, we set out to perform enteric bacteria sequencing in CFS, to compare it with the gut microbial composition of healthy controls and assess the differences, we try to identify potential markers that distinguish CFS patients from healthy controls, and also we assess the cognitive functions in both comparing groups and their correlation with microbial composition.

In the current study, we have noted that 143 (46%) enteric bacteria genera were present in CFS patients only. In addition, the gut microbial composition in the CFS was characterized by a lower abundance of the 20 most common types of bacteria compared to the HCs group, and the observed number of OTUs overall was lower in CFS than in HCs. Decreased diversity of major microbial bacteria phyla have been observed in previous studies[13–15]. Giloteaux et al. reported a lower overall diversity of gut microbial composition in CFS cases, compared to healthy controls[15,16]. Mandarano et al. detected lower *Eukaryotic* diversity of the gut microbial composition in CFS[15]. Some studies have been conducted to determine the overall gut microbiome composition, but more often they have only focused on the designation of a single differentiating species, to find out the CFS marker. Nagy-Szakal et al. reported a rise in *Bacteroides*, with the exclusion of *Bacteroides vulgatus*, in CFS patients[16]. Lupo et al. observed decreased abundance of *Lachnospiraceae* and an increased abundance of *Bacteroides* and *Phascolarctobacterium* in CFS patients, in comparison to HCs[1]. Fremont at al. noted the increased abundance of *Lactonifactor* and *Alistipes*, and decreased abundance of *Firmicutes* in CFS patients[17]. These previous observations are hard to compare to current study results, but it is clear that microbial composition in CFS patients is changed in comparison to healthy ones and it requires more investigations in the future.

In the current study, we obtained a higher relative abundance of *Bacteroidetes* and a lower level of *Firmicutes* in CFS, compared to HCs, which is consistent with previous reports. A decrease in abundance (the number of bacteria in the analyzed specimen) and diversity (the variety of bacteria in the analyzed specimen) of *Firmicutes* bacteria and a higher number of *Bacteroidetes* was detected in Giloteaux et al. studies[13]. Also, a lower *Bacteroides/Firmicutes* ratio, accompanied by an increase in *Enterobacteriaceae*, was previously observed in some studies, suggesting a complete reshuffling of the gut microbial composition[1,17].

The quantitative composition of the gut microbial composition is extremely variable and depends on many external and internal factors. The metabolic function of the gut bacteria is more permanent[18]. The functional core of microbial composition provides a specific metabolic and molecular profile, is resistant to interfering factors, and has the ability to return to a healthy functional profile, seems to correspond better with a notion of healthy microbial composition[19]. Based on current knowledge, we are not able to speculate about the impact of a lower enteric bacteria abundance in CFS patients on symptoms severity; further research is necessary.

In the current study, significant differences in beta-diversity of the enteric bacteria in CFS, in comparison to HCs, were noted. The three most discriminating ASVs: ASV 191, ASV 44, and ASV 75, were identified as significantly more abundant in the healthy control group compared to the patient group. Previously, the specific gut bacteria species that could be potentially important in differentiating CFS cases and suggested that they could be used as future CFS biological markers were reported in a study conducted by Kitami et al.[20]. Kitami et al. assessed 26 molecular markers to investigate which of them are the most differentiating for CFS and can be used as biological markers, and they determined that monocyte number, microbial abundance, and lipoprotein profiles appeared to be the most informative as CFS markers[20]. They assessed that lipoprotein and microbial changes occur early during illness, suggesting that these markers can be examined in a larger cohort for potential biomarker application[20]. Kitami et al. noted that the three most characteristic bacterial species were *Coprobacillus*, *Eggerthella*, and *Blautia*[20]. Although exact genera differ, on functional level this observation ispartially consistent with our study results. *Oscillibacter* and *Roseburia* (identified in our study as more abudant in HCs) are recognized butyrate-producing genera that contribute to gut health by exerting anti-inflammatory effects and maintaining mucosal integrity[21]. Similarly, *Ruminococcus_2* and *Blautia* have been implicated in fermenting dietary fibers into short-chain fatty acids (SCFAs), particularly acetate and butyrate, which play key roles in energy metabolism and immunomodulation within the host[22,23] Together, these bacteria support intestinal homeostasis and may influence systemic inflammation, relevant to conditions such as ME/CFS. Previous analyses report ME/CFS cases showing particularly depleted levels of major butyrate producers like *Roseburia* and *Ruminococcus*[13,24]. These taxa are critically important for colonic health and anti-inflammatory processes; reductions in their abundance have been repeatedly correlated with heightened symptom severity and metabolic abnormalities in CFS cohorts. *Oscillibacter*, though less frequently profiled, is increasingly recognized in recent metagenomic studies as a component altered in CFS and other inflammatory- or fatigue-related conditions[25,26]. Consistent network-based and causal inference approaches highlight that these compositional differences extend beyond isolated taxa to involve broader metabolic and interactional rewiring within the gut ecosystem[27].

The present identification of lower abudance of *Ruminococcus_2* and *Roseburia in CFS patients* aligns tightly with mechanistic hypotheses around impaired butyrate synthesis, increased gut permeability, and pro-inflammatory signaling seen in ME/CFS. Notably, these patterns are also observed when ME/CFS is parsed from related conditions, supporting the idea that these microbial shifts are a defining feature of the syndrome rather than a generalized consequence of chronic illness[13,28].

Given the convergence of these lines of evidence, our findings further support the existence of a distinct gut microbiome signature in ME/CFS[29]. Previous studies have suggested that the ME/CFS-associated gut microbiota is characterized by a reduction in butyrate-producing bacteria. Consistent with this, our study observed decreases

in *Roseburia* and *Ruminococcus*, key butyrate producers. Additionally, we identified significant differences in beta-diversity compared to healthy controls, with involvement of taxa such as *Oscillibacter*, although the precise role of these microbes in ME/CFS pathophysiology requires further investigation.

In the current study, we observed a significant interaction between the overall gut microbial composition and the indicators of the executive function: Trail Making Test part B (measuring cognitive flexibility and executive control), and the difference between scores in parts B and A (representing set shifting) indicating that cognitive function is related to gut microbial composition in both healthy controls and patients suffering from CFS. We also observed that individuals with worse (i.e. higher) scores on TMT had similar gut microbial composition, regardless of the group. What is interesting, a correlation of TMT B score was observed in the CFS with 8 genera, and in all of those relationships, the higher the abundance, the better cognitive function. In comparison, 29 correlations were noted between TMT B score and genera in the HCs, and 9 of them, the higher the abundance was related to the better the cognitive function, while in 20 correlations, the higher the abundance, the worse the cognitive function was. In both CFS and HCs, *Ruminococcaceae_UCG*–014 higher abundance was related to better executive cognitive function. In contrast, the *Ruminococcaceae*_UCG-010 was related to worse executive function in the control group and better executive function in CFS patients. The phylum *Firmicutes*, genus *Ruminococcaceae*_UCG-014, is known to make short-chain fatty acids (SCFA) and to live autochthonously in the colon and cecum[30]. Presumably, metabolites of *Ruminococcaceae*_UCG-014 might be related to better cognitive function. The endo-1,4-beta-xylanase and cellulase genes found in most *Ruminococcaceae* species are in charge of breaking down the various cellulose and hemicellulose components of plant material[31]. In our study, higher *Faecalibacterium* was related to better executive function in CFS patients. In line with our results, the quantity of *Faecalibacterium* was found to be favourably connected with cognitive performance in healthy individuals as opposed to those with moderate cognitive impairment (MCI) and Alzheimer's disease (AD) patients[32]. Somewhat in contrast to our results, *Christensenellaceae*_R-7_group was higher in the MCI patients than in the group without MCI[33]. In the current study, higher *Roseburia* was related to better executive function in CFS patients. Increase of *Roseburia* intestinal in animals model of colitis was related to the improvement of the gut-brain axis[34]. In the current study, a relationship between a better TMT B score and higher *Ruminiclostridium*_6 was observed. In a study based on fiber supplementation, an increase in *Ruminiclostridium*_5 and a modest improvement in cognitive function were noted[33].

It remains unclear whether changes in the nervous system are primary or secondary to enteric dysbiosis. The idea that the gut microbial composition can modulate cognitive function has received support from both rodent and human research[35]. Numerous rodent studies have consistently shown the impact of gut microbial composition manipulations on the hippocampal-dependent memory function (correlated with BDNF and neurogenesis) and cognitive flexibility (possibly linked to an increase in the cortical glutamatergic function)[35]. Human researches, although less abundant, points towards the same conclusion. A few studies linked the application of pre-and probiotics with an improvement in cognitive functions, including memory[36–38], verbal learning and social-emotional cognition[37], and emotional attention[39]. Possibly most relevant to this study is the investigation into the links between obesity, the microbial, and cognition, which displayed a relationship between the gut microbial composition with speed, attention, and cognitive flexibility in the Trail Making Test A[40]. Our findings add to the increasing body of evidence supporting the link between the gut microbial and cognitive function in CFS. While more research is needed, the direction in which this area is developing is promising, especially as it creates a new field for potential therapeutic interventions.

In the current study, we have noted qualitative and quantitative differences in networks made based on the gut microbial in the CFS vs. HCs groups. In addition, the highest AUC obtained by the neural network (multilayer perceptron) was 0.94, which could be considered an excellent discriminatory performance[41]. Three of the ASVs with the best discrimination capability were ASV 3979, ASV 5362, and ASV 272 according to SHAP values. In the current study, both supervised and non-supervised approaches were used for patient classification and phenotyping, respectively. All data collected in the current project was tabular. Tabular data was described as an "unconquered castle" for "deep" artificial intelligence (AI) models[42]. At the moment, gradient-boosted decision trees and other "shallow" models are regarded as the gold standard in an application for tabular data[43]. In the current study, multiple ML models were applied. It should be underlined that microbial composition data has its specific features, such as the presence of a large number of zeros[44] and multivariate character. The combined presence of those two factors might potentially explain why "shallow" models with specific regularization techniques, as applied in the current study, might be characterized by a relatively fair performance in comparison to the rest of the models applied in tabular data. ML models have potential shortcomings related to their low interpretability[45]. The explainable artificial intelligence (XAI) approach is trying to overcome this issue. In the current study, we have applied SHAP, which might serve both for global explanations and local interpretations[46,47]. It might be concluded that the currently applied model tended to overfit, which might limit its availability in discriminating gut microbial composition in CFS vs. HCs in further studies.

Study limitations

The current study significantly contributes to filling some of the gaps in knowledge about CFS pathophysiology. We used 16 S rRNA sequencing, which allows for the accurate identification of most microorganisms even at the ASV sequence level. However, the study has some limitations. CFS diagnosis was based on the Fukuda Criteria, which might serve as a limiting factor. Older criteria for CFS, such as the Fukuda criteria (also known as Centers for Disease Control and Prevention (CDC) 1994 criteria)[48] do not require the presence of post-exertional malaise (PEM), unlike more recent ME/CFS diagnostic criteria such as the Canadian Consensus Criteria (CCC)[49], International Consensus Criteria (ICC)[2] and NICE criteria[50]. Therefore, further studies should investigate how gut microbial composition and function relate to the intensity and fluctuations of PEM, as it is a cardinal symptom of ME/CFS. The study and control groups were small, and patients with severe

and very severe symptom intensity were not included in the study, due to their limitations (bed-ridden and house-bounded patients could not engage in the study protocol). Applied classifiers, including the MLP neural network, achieved high performance. However, despite applying regularization methods and setting a maximum of 200 iterations (epochs), there is still a risk of overfitting. This might mean that the applied classifier has limited generalizability when applied to data obtained from other samples. On the other hand, the application of 10-fold cross-validation in the current study should help provide a more reliable estimate of the model's performance by reducing variance and bias in the evaluation, thereby offering a better indication of how well the model generalizes to unseen data. These limitations should be considered while designing further experiments. Recently, the need for specialized, validated statistical frameworks and user-friendly tools that can handle the intrinsic features of microbial composition datasets and provide reliable power/sample size estimates for various study designs (e.g., case-control, longitudinal) is emphasized[51,52]. The network analysis in the current study provided visual insights but was based on correlation-based methods that inherently cannot determine causality or the direction of interactions between taxa. To overcome these limitations, future studies might employ more sophisticated techniques like SPIEC-EASI[53] or CoNet[54], which are designed to infer more reliable microbial association networks by accounting for compositionality and reducing spurious correlations. These approaches could enhance the biological interpretability and robustness of inferred microbial interactions. A limitation of this study is that participants' history of antibiotic and probiotic use, as well as recent episodes of diarrhea, were neither collected prior to participation nor incorporated into the data analysis. The absence of this information limits the ability to control for these variables, which may affect the interpretation and generalizability of the findings. Overall, caution must be exercised when interpreting observed associations between gut microbial composition and cognitive function causally, as potential confounding factors such as diet, medication, and lifestyle may influence these relationships.

Methods

Patients

Sample size calculation was made using GLIMMPSE 3.0.0 online available calculator for General Linear Mixed Model Power and Sample Size (available at: https://glimmpse.samplesizeshop.org/#) as described previously[55]. It showed that at least 50 participants were included in total into the analysis when assuming a dropout rate of 11%. Two hundred fifty patients, who identified themselves as fatigued, were referred to the study by their general practitioner, neurologist, or psychiatrist. The inclusion criteria for the study were as follows: (1) age between 25 and 65 years, men and women, (2) fatigue for more than 6 months, due to unknown causes, (3) at least four additional symptoms: malaise after exertion, impaired memory and/or concentration, headache, unrefreshing sleep, tender lymph nodes (cervical or axillary), sore throat, muscle or joint pain. The exclusion criteria reflect Fukuda's criteria for diagnosing CFS, including the presence of an illness that might trigger chronic fatigue (e.g., cardiovascular disease, autoimmune disease, or psychosocial stress). The pre-test health state assessment included: basic psychiatric and neurological, clinical examination. Physicians experienced in CFS diagnosis confirmed the inclusion and exclusion criteria and checked whether an extensive physical examination and to exclude any chronic disorder that might explain fatigue. After undergoing the initial eligibility assessment, 180 were excluded as they did not meet the Fukuda criteria, 30 as they had an underlying psychiatric illness, and 8 with another diagnosis commonly related to fatigue (untreated hypertension) or fatigue was not the primary complaint, leaving a sample of 32 patients who met the criteria for CFS. For details, see[55]. Seven patients with CFS and 7 HCs did not provide stool samples, despite at least two reminder phone calls (Figure S6), and were excluded from the analysis. The remaining sample, included in the analysis, consisted of 25 patients with CFS and 16 healthy controls. Healthy controls were free from any chronic disorder, which was confirmed during the face-to-face examination. Gut microbial composition was compared in 25 patients with CFS (20 females) to 16 HCs (10 females). The research process is shown on a flow chart, available online as Supplementary Figure S6.

The study was conducted according to the guidelines of the Declaration of Helsinki and obtained ethical approval from the Ethics Committee, Ludwik Rydygier Memorial Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University, Toruń (KB 660/2017). Informed, written consent was obtained from all subjects involved in the study.

Measures

Assessment of cognitive function

Cognition was assessed with the Trail Making Test (TMT), a neuropsychological task probing executive function, with an assessment of such processes as attention, inhibitory control, task switching, visuospatial skills, and working memory[56]. The test was performed during face-to-face evaluation by trained staff using paper and pencil version. It consists of parts A and B. Trail Making Test part A (TMT A) requires connecting 25 encircled numbers in sequential order. Trail Making Test part B (TMT B) requires connecting 25 encircled numbers (from 1 to 13) and letters (from A to L) in, respectively, ascending numerical and alphabetical order while alternating between numbers and letters (1-A-2-B, etc.). The outcome of TMT A and B is the time to complete the task, measured in seconds. TMT A probes visual search and motor speed skills[57], while TMT B is considered to test cognitive flexibility and executive control as well[58–60]. The difference between TMT B and A scores (TMT B-A) is considered to be an indicator of executive function. The lower the score of TMT A, TMT B and TMT B-A, the better visual search and motor skills, cognitive flexibility and executive control and executive function, respectively.

Intestinal microbial composition assessment

Gut microbial composition was analyzed using stool samples. Patients were instructed to collect fecal samples in the morning immediately after waking up and to place them directly into a sterile container. The samples were then transported to the laboratory, where they were stored at − 80 °C until analysis. There is an increasing amount of data that shows the superiority of analysis based on amplicon sequence variant (ASV)[61], therefore the analysis was based mainly on the ASV. If results were obtained based on the operational taxonomic unit (OTU), then it is denoted in the description. The concurrent use of OTU and ASV methodologies can be justified by their complementary strengths: OTUs, traditionally used for alpha-diversity, maintain comparability with legacy ecological metrics and capture broader richness patterns despite clustering similar sequences, while ASVs provide higher resolution and reproducibility, making them better suited for beta-diversity analyses and classification models that require fine-scale discrimination of microbial variants. Comparative studies have demonstrated that although OTU clustering tends to underestimate species diversity relative to ASVs, both methods yield broadly similar patterns of relative richness and community composition across diverse environments, supporting their complementary use[62,63]. Differences in taxonomic resolution and detection of certain taxa have been noted, highlighting the importance of clearly articulating the rationale for each approach and performing validation or comparative analyses to demonstrate consistency and complementarity of results, thereby reducing reader confusion and enhancing robustness of ecological conclusions[62,64,65]. The dual approach leveraging both OTU and ASV pipelines to provide a comprehensive ecological assessment is supported by recent intestinal bacteria informatics literature, which highlights that while OTU clustering methods offer comparability with legacy datasets and broader ecological indices, ASV methods deliver higher resolution, improved reproducibility, and more precise microbial identification essential for detailed beta-diversity and classification analyses[62,64,65].

Gut microbial composition diversity assessment

The enteric bacteria diversity was determined by sequencing 16S rRNA gene fragments. For gut microbial composition sequencing, DNA was isolated from fecal samples by bead-beating (Powerlyzer, Mo-Bio) in combination with flocculation and silica columns. V3-V4 bacterial 16S rRNA gene fragments were amplified and transformed into Illumina sequencing libraries. Libraries for sequencing on the MiSeq (Illumina) were prepared in two rounds of PCR, the first using primers specific to the 16S rRNA genes carrying the M13 and M13R sequences at their 5' ends and the second using the M13 and M13R primers carrying the adapter sequences (P5 and P7) and MID (molecular identifier) to distinguish sequences from individual samples. After sequencing, the reads were denoised (BayesHammer), assembled (Pandaseq), and processed in Mothur[66] as described by Thiem et al.[67].

Quantitative assessment of the gut microbial composition and data pre-processing

The number of bacteria in all samples was tested by qPCR (on a LightCycler 480 apparatus) using a pair of primers B969f and B1072r, amplifying the 16 S rRNA gene fragment from over 90% of known sequences (data by SILVA). Samples were hybridized with the fluorescently labelled (Rox) probe B969f and analyzed on the BD Aria III cell sorter at 488 nm excitation and fluorescence measurement at 610–620 nm. Sequencing reads were processed as previously described[67]. Briefly, sequencing reads were denoised, merged, and checked for the presence of chimeras using the dada2 package[68]. Then, reads were classified using the SILVA database[69]. Operational taxonomic units (OTUs) were then constructed, and a common OTU table was defined using the Mothur package[66]. Ecological analyses were performed using the R software using functions implemented in the packages vegan[70] and GuniFrac[71]. The unweighted UniFrac distance matrix was generated using the GUniFrac function from the OTU sparse table and tree. All methods were performed according to relevant guidelines and regulations. The ASV count data were preprocessed by first removing negative control samples and ASVs classified as mitochondria or chloroplasts, to control for contaminants. Table S3 presents taxon names and their corresponding ASV numbers (Table S3). The dataset was then rarefied 100 times at a sequencing depth of 3000 reads per sample, with the results averaged and rounded to generate normalized count tables for the full dataset as well as for samples collected before and after cryopreservation. Sample metadata and count data were matched and sorted to ensure alignment. The repeated rarefaction approach was used to address uneven sequencing depth and reduce the impact of zero inflation. Average sequencing reads per sample was 11802.67.

Statistical analysis

Alpha- and Beta-diversity were assessed. Alpha-diversity is defined as the variety of samples representing a given community, or habitat. Beta-diversity is a measure of the variation in the occurrence of species in an environmental gradient or between different communities. Between-group differences (CFS vs. HCs) in quantitative variables describing the characteristics of the groups were assessed using the Mann's U test -Whitney was used. The cut-off level of significance was $p < 0.05$.

The significance of between-group differences was tested by the PERMANOVA method (adonis2 function) using 999 permutations. In addition, Distance-based ReDundancy Analysis (dbRDA) was conducted based on the Bray–Curtis dissimilarity. sPLS-DA (sparse version of the Partial Least Squares Regression-Discriminant Analysis) was used to distinguish the microbial composition of CFS patients from controls[72]. sPLS can be used to simultaneously reduce dimensions and select variables that differentiate subgroups of patients[73]. Using sPLS-DA, features in the data can be selected to classify samples with the highest predictive value or discriminating feature[72]. The results of the analyses were presented in the form of bar graphs containing two coordinate axes and columns whose height illustrates the value differentiating the CFS compared to the HCs. To summarize the strength and direction (negative or positive) of the relationship between gut microbial composition and results of TMT B, Spearman's rank correlation coefficient was used. Metacoder package was used to perform the network tree heatmap[74].

Analysis of the classification of CFS patients compared to healthy volunteers, using machine learning (ML) methods, was performed using the Orange program[75]. The following ML methods were used: Logistic Regression (with Lasso regularization), Logistic Regression (with Ridge regularization), Random Forest Classification, XGBoost (eXtreme Gradient Boosting), CatBoost (Categorical Boosting), XGBoost RF (Random Forest), AdaBoost (Adaptive Boosting), Neural Network (multilayer perceptron), SVC (Support Vector Classification). The strength of the regularization in Logistic Regression models was set to C = 0.7. In the Random Forest Classification, the number of trees was 1000, with the principle of not splitting subsets smaller than 5. In XGBoost, CatBoost, and XGBoost RF the number of trees was 1000, learning rate = 0.3, Lambda = 1, limit of the depth of individual trees was 6. In AdaBoost, the number of estimators was 50, the learning rate was 1, classification algorithm was SAMME.R. In the SVC, a linear kernel was used, C = 1, epsilon = 0.1, numerical tolerance value = 0.001, and a maximal number of iterations was set to 100. The Neural Network (multilayer perceptron) consisted of 5 layers (consisting of 1000, 500, 200, 100, and 100 neurons, respectively) The activation method was ReLu, the optimization method was Adam, the regularization value $\alpha = 0.0001$, and a maximal number of iterations was set to 200. Model performance evaluation was done using stratified 10-fold Cross validation. Reported scores include AUC (Area Under The Curve), ROC (Receiver Operating Characteristic), CA (classification accuracy compared to controls), F1, precision, recall, and MCC (Matthews correlation coefficient)[76]. The model's discriminatory performance was assessed based on AUC, as it is a metric widely used in clinical research and easy to interpret. An AUC of 0.5 suggests a non-discriminatory model (i.e., the ability to diagnose patients with and without a disease or condition based on the test), 0.7 to 0.8 is considered acceptable, 0.8 to 0.9 is considered excellent, and greater than 0.9 is considered outstanding[41]. We also reported the approximate Shapley Additive exPlanations (SHAP) value to identify important group-discriminatory bacteria for the top-performing model.

Network analysis was performed using the NetComi package[77]. A network constructed based on enteric bacteriaASVs from the CFS was compared to the HCs. The zero-inflated ASV count data were preprocessed using multiplicative zero replacement (zeroMethod = "multRepl") to impute zeros with small positive values, enabling subsequent log-ratio transformations. Following this, a centered log-ratio (CLR) transformation (normMethod = "clr") was applied to account for the compositional nature of microbial composition data by normalizing counts relative to their geometric mean within each sample. This combination of zero replacement and CLR transformation helps mitigate biases and spurious correlations inherent in compositional data, thereby improving the validity of downstream SparCC-based network inference. Additionally, taxa filtering based on highest variance retained the 40 most variable taxa to reduce noise. The Spearman correlation method with a threshold of 0.3 was used to calculate the relationship between the values of individual ASVs. The specified threshold served as the method used for sparsification (selecting edges that are connected in the network). The following methods were used for the statistical analysis of differences between the networks generated based on microbial composition data in the group of CFS patients compared to controls: Jaccard index, whose value ranges from 0.00 (networks are completely different to 1, which means that they are completely equal). The following global network parameters were compared: degree, betweenness centrality, closeness centrality, eigenvector centrality, and hub taxa.

Data availability

The dataset is available on-line (https://repod.icm.edu.pl/dataset.xhtml?persistentId=doi:10.18150/2XR47C).

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Author contributions

J.S., and P.Z. participated in conceptualization (create ideas and overarching research goals) and methodology. S.K., J.S., Ł.S., P.Z. gathered data, S.K. analyzed data, M.P.J., H.T., S.K., B.R.G., J.S., B.J.-G., M.M., K.J.M., Ł.S., and P.Z. drafted manuscript. All authors read and approved a final version of the manuscript.

Founding sources

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Declarations

Competing interests

Sławomir Kujawski serves as an Editor in Scientific Reports.The authors declare no other competing interests.

Ethics declarations

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee, Ludwik Rydygier Memorial Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University, Toruń (KB 660/2017). Informed, written consent was obtained from all subjects involved in the study.

Additional information

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