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POTENTIAL ROLE OF MICROBIOME IN CHRONIC FATIGUE SYNDROME/MYALGIC ENCEPHALOMYELITIS (CFS/ME)

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ABSTRACT

The Chronic Fatigue Syndrome (CFS), also known as Myalgic Encephalomyelitis (ME), is a severe debilitating systemic disease of unknown etiology that involves multiple systems including the nervous, immune, endocrine, digestive, and skeletal systems, accompanied by dysfunction of energy metabolism and cellular ion transport. CFS/ME patients show a persistent, unexplained fatigue accompanied by a number of secondary symptoms including cognitive dysfunctions, unrefreshing sleep, post-exertional malaise.

Due to the great heterogeneity of CFS/ME populations, to date there are not specific biomarkers and diagnostic tests for this pathological condition. Some features of the syndrome, such as the "*relapsing-remitting*" symptoms, the higher prevalence in the women and a persistent immune activation, suggest a similarity with autoimmune conditions.

Subjects with CFS/ME also suffer gastrointestinal symptoms, already described in Irritable Bowel Syndrome (IBS), often correlated with a change in the intestinal microbial composition. The oral and gut microbiota are the most complex microbial community in the human body and it is well known that both are able to display signatures associated with pathologies. Changes in the intestinal bacterial composition have been detected in metabolic diseases, intestinal disorders, autoimmune conditions, cancer and in several neurological disorders, highlighting that there is a strong correlation between dysbiosis and the pathological condition and therefore, changes in the composition and function of the microbiota could be somehow implicated also in the pathogenesis of CFS/ME. Although some studies have reported alterations of intestinal and oral microbiota in CFS/ME, the relationship between the bacterial composition and the pathogenesis of this syndrome has not yet fully demonstrated.

For these reasons, the present research aimed to investigate the features of intestinal and oral bacteriome in adult patients with CFS/ME in order to assess whether any changes in the microbial composition may be somehow involved in the pathogenesis of CFS/ME and to determine whether any observed differences could be useful in the future for the identification of diagnostic biomarkers.

To this purpose, in this study 105 volunteers were enrolled: 35 CFS/ME patients, diagnosed according to Fukuda's criteria, were investigated and compared with a population of relatives without CFS/ME living with patients and a healthy control group chosen outside the patients' families. To investigate the oral and intestinal microbiota in CFS/ME subjects,

a metagenomic approach was applied. This approach involved the direct isolation of total DNA from fecal and salivary samples followed by the selective amplification of bacterial DNA using specific universal primers for the hypervariable regions V3-V4 of the 16S rRNA prokaryotic gene. Amplicons were sequenced using NGS high-throughput platform (MiSeq-Illumina); bioinformatic and statistical analysis, using dedicated softwares (Mothur, R), were applied for comparing, analyzing and interpreting sequencing data.

Based on the results of intestinal bacterial composition in CFS/ME patients, a pilot study was conducted on a subgroup of CFS/ME patients which belonged to a same cluster at family level to evaluate whether the metabolic profile of CFS/ME patients differed from those of their relatives and external controls. The metabolic analysis was performed on fecal samples by an Ultra Performance Liquid Chromatography (UPLC) interfaced with a high-resolution Q-ToF mass spectrometer (MS).

The results of the present study showed significant variations in both the intestinal and oral bacterial composition between CFS/ME patients, their relatives and external controls, due to changes in the relative abundances of several bacterial taxa. Interestingly, the relatives, in the most cases, showed intermediate prevalence values.

Considering the fecal bacterioma, the analysis at taxon level showed a reduction of *Firmicutes* and, on the contrary, a significant increase of *Bacteroidetes* in CFS/ME patients in comparison with the non-CFS/ME groups.

The reduction of *Firmicutes* and the increased proportion of *Bacteroidetes* observed in CFS/ME patients and in their relatives was mainly ascribed to members of *Clostridiales* and *Bacteroidales*, respectively. Within *Clostridiales*, several families declined, with *Lachnospiraceae* showing the greatest decrease. A significant reduction of genus *Anaerostipes* was observed in CFS/ME patients and in their relatives. In addition, *Phascolarctobacterium* faecium and unclassified *Ruminococcus* were significantly increased only in CFS/ME patients compared to external control group.

A significant increase in *Bacteroidaceae* and *Barnesiellaceae*, particularly *Bacteroides* and *Barnesiella* genera, was observed both in CFS/ME patients and their relatives. *Bacteroides vulgatus*, unclassified *Bacteroides*, *Bacteroides uniformis, Bacteroides ovatus* and unclassified *Barnesiella* resulted significantly more abundant in CFS/ME patients and in their relatives.

The analysis of salivary bacterioma revealed a greater species richness than that observed in feces, although few differences between the experimental groups were observed. The comparison among CFS/ME patients, their relatives and external controls pointed out major differences for *Actinobacteria*, which significantly increased in CFS/ME patients. The

increased abundance of *Actinobacteria* observed in CFS/ME patients and, to a minor extent, in their relatives was associated with a higher prevalence of *Actinomycetales*. Within this order a significant increase of *Micrococcaceae*, particularly *Rothia sp.*, was observed only in CFS/ME patients. Two pathogenic species belonging *Rothia* genus were identified, *Rothia dentocariosa* and *Rothia mucilaginosa*, although the statistical significance was obtained only for *Rothia dentocariosa*.

The fecal metabolic profile in a subgroup of CFS/ME patients resulted to be different compared to that of their relatives and external controls, although the differences were not statistically significant. An overall increase of SCFAs and indole derivatives was observed in the CFS/ME cohort in comparison with the non-CFS/ME groups, suggesting an increase in the fermentation processes.

In light of the results obtained, CFS/ME patients showed alterations in the composition of both the fecal and salivary microbiota, with more marked differences observed in the gut. While confirming the results of previous studies (Fremont *et al.*, 2012; Shukla *et al.*, 2015; Giloteaux *et al.*, 2016), these results add new information and support the autoimmune hypothesis for CFS/ME condition in that in this study the intestinal microbial profile recorded in CFS/ME patients is consistent with that reported for autoimmune conditions, such as Chron's disease (Manichanh *et al.*, 2006), ulcerative colitis (Maukonen *et al.*, 2015) and Systemic Lupus Erythematous (Hevia *et al.*, 2014).

In CFS/ME patients, the decrease in the abundance of several butyrate-producing bacteria belonging to *Lachnospiraceae* may result in the alteration of the integrity of the intestinal barrier and in a reduced protective action against gut inflammation. The increase of *Bacteriodes* species, some of which are able to damage the intestinal barrier by means of their virulence factors, may compromise the permeability of the intestinal barrier, resulting in a "*leaky gut*", and promote bacterial translocation in the bloodstream, causing an abnormal systemic inflammatory response.

As oral microbial communities are closely connected with the intestinal microflora and influence its composition, the higher prevalence in CFS/ME patients of oral opportunistic pathogens (i.e. *Rothia dentocariosa*) able to cause infections in several body sites, may alter the composition of their gut microbiota and dysregulate their immune tolerance.

The gradual increase or decrease of most bacterial taxa observed in CFS/ME patients and in their relatives compared to external controls, suggest the presence of a modified microbiome profile also in patients' relatives, affected by genetic and environmental factors (i.e. diet and/or environmental pollution).

The metabolic analysis carried out on a subgroup of the three experimental populations allowed to record some differences in the fecal metabolic profiles of CFS/ME patients. Although the observed differences were not statistically significant, some data appear very interesting and deserve to be deeper investigated. These results, if confirmed by using a larger cohort, may lead to a better understanding of the relationship between metabolic changes and CFS-related immunological and cognitive dysfunctions.

In conclusion, this work represents the first microbiological study carried out on an Italian population of CFS/ME by applying the NGS techniques and including the relatives of CFS/ME patients. To obtain data that are truly representative of the pathological condition it will be of crucial importance to analyze a larger cohort of patients and perform longitudinal studies using the same workflow.

Further studies are needed to better understand whether the alteration of the microbiota is a cause or a consequence of the onset of CFS/ME and if the alterations of the microbiota are related to any of the several secondary symptoms.

Despite evidences of altered composition of the intestinal and oral microbiota in CFS/ME, a specific microbial signature attesting a pathogenic role of the microbiota in CFS/ME has not yet been identified. If our results will be confirmed by larger studies, the differences detected in the microbial and metabolic profiles of CFS/ME patients may be used as markers for a more accurate diagnosis of the syndrome and for the development of specific therapeutic strategies.