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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 accompained 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-producting 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.

1. INTRODUCTION

The thesis work reported here aims to investigate the intestinal and oral bacterioma of adult subjects affected by Chronic Fatigue Syndrome (CFS). CFS is a debilitating multi-systemic disease that mainly affects young women aged 25 to 40 and is characterized by severe debilitating fatigue and a combination of symptoms such as problems in concentration and in short-term memory, sleep disorders and musculoskeletal pain. (IoM, 2015)

The problem of health and economic management of patients affected by this pathology has aroused a growing interest by health institutions in different countries. In Italy, this pathological condition is poorly known and diagnosed. However, the Italian National Agency for Regional Health Services (AGENAS) has developed the first guidelines on CFS that collect the current knowledge on both the etiology and pathophysiology of this disease. The lack of biomarkers and specific diagnostic tools for this syndrome causes long delays in its diagnosis and consequent serious discomforts for patients. Although various causal hypotheses have been formulated (infectious, immunological, cognitive, genetic), to date the etiology of CFS is still unknown. Subjects with CFS also report gastrointestinal symptoms, already described in Irritable Bowel Syndrome (IBS), and often correlated with a change in intestinal microbial composition (dysbiosis). Indeed, one of the current hypothesis is that in these subjects occurs an alteration of the intestinal microbiota: in particular, the replacement of some bacterial species would induce signals able to alter the existing equilibrium (mutualism and tolerance).

The analysis of the oral and intestinal bacterial communities is motivated by the fact that they 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 (Abe *et al.*, 2018; Shengtao *et al.*, 2019).

Changes in the biodiversity of microbiota has been observed in several diseases, suggesting a strong correlation between dysbiosis and pathological conditions. Therefore, changes in the composition and function of the microbiota could be somehow implicated also in the pathogenesis of CFS. Today the use of new culture-independent methodological approaches and the availability of last-generation sequencing technologies (NGS) allow to point out the wide diversity of microbial communities - and thus the effects of community changes on human health - more effectively than in the past decades. In this study, the oral and intestinal bacterial composition of patients with CFS was analyzed and compared with that of two healthy control groups, of which one consisting of patients' family members. The study promoted by the Italian

Association of CFS patients (AMCFS-onlus), was carried out by applying the NGS analysis of prokaryotic 16S rRNA gene amplicons. Bioinformatic and statistical analyses allowed to identify the bacterial taxa and detect differences in oral and intestinal microbial communities between CFS patients and healthy subjects.

1.1 Chronic Fatigue Syndrome / Myalgic Encephalomyelitis

Chronic Fatigue Syndrome (CFS), also known as Myalgic Encephalomyelitis (ME), is a severe debilitating systemic disease that involves multiple systems, including the nervous (Chen *et al.*, 2008), immune (Fletcher *et al.*, 2010; Lorusso *et al.*, 2009), endocrine (Cleare, 2003), digestive (Lakhan and Kirchgessner, 2010), and skeletal (Jones *et al*., 2009) systems, and is associated to dysfunctions of both energy metabolism and cellular ion transport (Myhill *et al.*, 2009).
CFS/ME patients show a persistent, unexplained fatigue and a number of secondary symptoms including cognitive dysfunctions, unrefreshing sleep, post-exertional malaise, myalgia and joint pain, that negatively affect the quality of patients' life greatly affecting their routinary daily activities (IoM, 2015).
The CFS/ME was described for the first time after two outbreaks that occurred in the mid-1980s in Nevada, near Lake Tahoe, and in New York. In those occasions, hundreds of people showed symptoms very similar to those caused by flu that lasted for an unusually long time and were characterized by unexplained muscle fatigue and cognitive abnormalities (Holmes *et al.*, 1988).

1.1.1 Epidemiology of CFS/ME

The first study on the epidemiology of CFS/ME was carried out by the Center for Disease Control and Prevention (CDC) in the late 1980s (Gunn *et al.*, 1993); the CFS/ME prevalence was assessed to be 4 – 8,7 ind. per 100,000 (Reyes et al., 1997). Larger studies estimated that between 800.000 and 2.5 million people are affected by CFS/ME in the U.S.A., resulting in a significant social impact and an annual economic cost of 17-24 billion dollars (Jason *et al.*, 2006; Jason *et al.*, 2008).
CFS/ME predominantly affects young adults, with a peak age of onset between 20 and 40 years, and is more common in women than in men, with a male/female ratio of 1:4 in some cohorts (Reyes *et al.*, 2003; Capelli *et al.*, 2010) Some recent investigations in the U.S.A. and U.K. indicate an average prevalence of 1.0% and 1.1% respectively for the two countries (Reyes *et al.*, 2003; Nacul *et al.*, 2011). In Italy, wide epidemiological studies have never been conducted,

and prevalence estimates are available only at regional-scale (Capelli *et al.*, 2015). A survey conducted between 2001 and 2010 by the Italian National Agency for Regional Health Services (AGENAS) assessed that CFS/ME prevalence in Italy should range between 0,1% and 0,2% (Lorusso *et al.*, 2014).

1.1.2 Etiological factors of CFS/ME

The etiology and pathogenetic mechanisms of CFS/ME are currently unknown. However, several hypotheses have been proposed since the second half of the 20th century (Fig. 1).

Infectious hypothesis. The infectious hypothesis is the oldest. A disease with the same symptomatology of CFS/ME was described in closed populations (convents and hospitals), suggesting a possible infectious etiology or the association of the onset of the disease with concurrent viral infections (Shelokov *et al.*, 1957; Albrecht *et al.*, 1964). In many CFS/ME patients the onset of the disease occurs after an infectious event with flu-like symptoms. A previous Epstein-Barr virus (EBV) infection was found in 11% of subjects with CFS/ME, with a high IgG antibody titer, indicating an episode of infectious mononucleosis (Hichie *et al.*, 2006). This virus, infects more than 90% of the global human population, but in most cases it is not diagnosed as it causes only slight flu symptoms. However, not all individuals with CFS/ME have a significant antibody titer for EBV and for this reason several studies have been carried out to identify other possible viruses as triggers of CFS/ME.

Several other viruses have been associated with the onset of CFS/ME including the Ross River virus (Hichie *et al.*, 2006), human herpesvirus-6 (Ablashi *et al.*, 2000), parvovirus B19 (Fremont *et al.*, 2009), and human enteroviruses (Chia, 2005). Nonetheless, EBV remains the most frequently recorded virus. Studies conducted on murine models investigated the role played by EBV proteins expressed during viral replication in the induction of the disease. In mice, the stimulation of the immune system through dUTPase induced immuno-modulating effects similar to the clinical symptoms observed in subjects with CFS/ME. These effects included the lowering of physical activity and body mass index, which were correlated to the increasing production of inflammatory cytokines, and increase in IFNγ synthesis and activity of Natural Killer cells (Glaser *et al.*, 2005; Glaser *et al.*, 2006).

Although a specific infective causal agent of the disease has still not been identified, the interaction of some infectious agents in a predisposed individual may induce the dysregulation of the immune system, causing the ineffective containment of the pathogen, persistence of chronic inflammation and damage to the Central Nervous System (CNS).

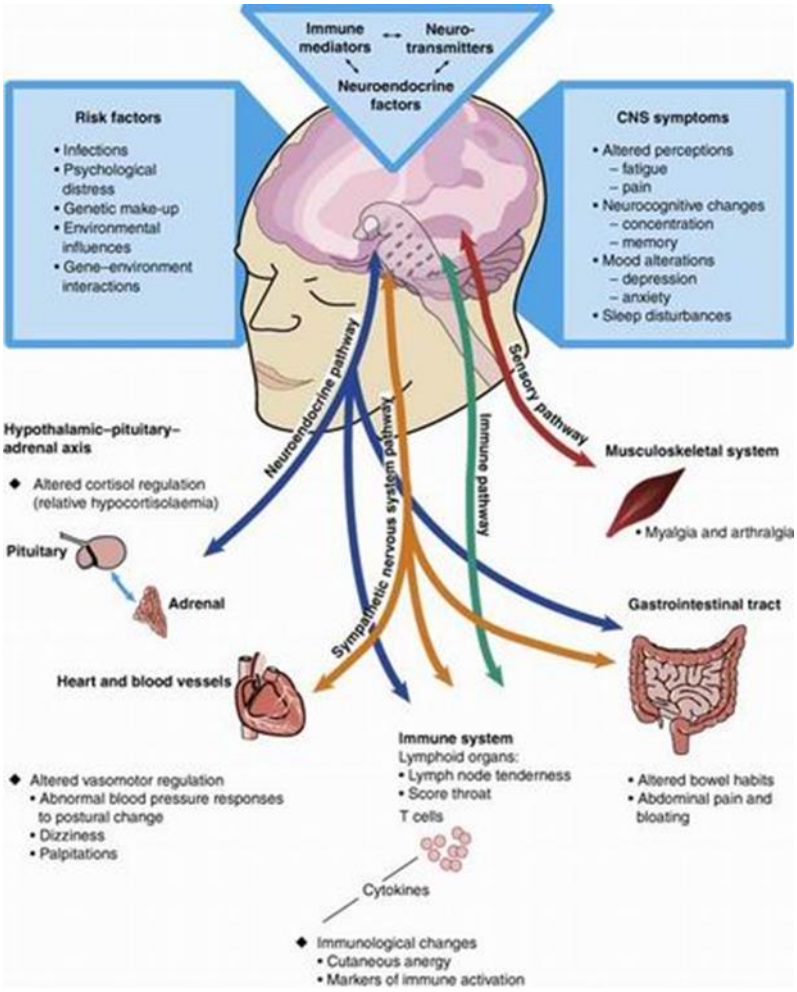

Figure 1. Physiology and Pathophysiology of CFS/ME. *Scheme of physiogical mechanisms and risk factors involved in CFS/ME pathogenesis. (Carlisle S., & Thompson, J., 2014. Imagine retrieved from https://healthlifemedia.com/healthy/what-is-chronic-fatigue-syndrome-cfs/)*

Immunological hypothesis. This hypothesis is based on the observation that CFS/ME could be the consequence of an abnormal behavior of the immune system in response to an infectious stimulus. CFS/ME can occur with inflammatory symptoms and is characterized by an "*abnormal*" immune response after physical exertion (Nijs *et al.*, 2014). Several non-specific immunological abnormalities have been described in CFS/ME: alterations of cell-mediated immunity, with a reduction in the activities of Natural Killer cells a strong increase in pro-inflammatory cytokines levels (IL-1β, IL-6, TNFα) (Blundell *et al.*, 2015; Horning *et al.*, 2015), and a decreased expression of Interferon-γ (IFN-γ) (Carlo-Stella *et al.*, 2006). These data support the hypothesis that the activation of inflammatory mechanisms occurs in subjects with CFS/ME (Glaser *et al.*, 2005).

Furthermore, some features of the syndrome, such as "*relapsing-remitting*" symptoms, the higher prevalence in women and persistent immune activation, suggest a similarity with autoimmune conditions. Consistently, the presence of antinuclear autoantibodies and

circulating immunocomplexes have been detected in some individuals with CFS/ME, supporting the hypothesis that CFS/ME could be an autoimmune disease (Skowera *et al.*, 2002). This hypothesis is supported by the results obtained by a clinical study which showed that a monoclonal antibody (Rituximab), that acts by depleting B cells (which, in their turn, produce autoantibodies), allowed a significant reduction in fatigue scores and a general improvement in health conditions of CFS/ME patients after 8 months of treatment (Fluge *et al.*, 2011).

Neuro-endocrinological hypothesis. The etiology of CFS/ME can be explained by the Microbiota-Gut-Brain Axis, which represents the physiological network between the microbiota, the Central Nervous System (CNS), the autonomic and enteric nervous system and the hypothalamic-pituitary-adrenal (HPA) axis (Demitrack *et al.*, 1991).
In healthy subjects the physical and emotional stress activates the HPA axis, stimulating the synthesis and secretion of hormones, particularly cortisol. Preliminary studies in subjects with CFS/ME have shown a cortisol production lower than normal (Demitrack, *et al.*, 1991). Because cortisol suppresses inflammation and the activation of cell-mediated immunity, a reduction in circulating cortisol could alter the control of these processes.
A defect in the synthesis and/or release of corticotropin-releasing hormone (CRH), involved in HPA activation, has been hypothesized to contribute to the development of fatigue in subjects with CFS/ME. Indeed, an alteration of CRH pathways was observed in various chronic diseases characterized by fatigue such as Systemic Lupus Erytematosus (LES), Rheumatoid Arthritis (RA), Multiple Sclerosis and fibromyalgia (Clauw and Chrousos, 1997; Swain, 2000). Most clinical features observed in patients with CFS/ME are similar to those found in patients with fibromyalgia, suggesting that the pathophysiological mechanisms may be the same (Aaron *et al.*, 2000).

Genetic hypothesis. Evidence supports the possibility that genetic predisposition may enhance the development of CFS/ME. First-degree relatives of CFS/ME patients are more likely to be affected by the same disease than unrelated individuals (Walsh *et al.*, 2001; Albright *et al.*, 2011). Studies on twins suggest that monozygotic twins are more prone to be infected than heterozygous ones (Buchwald *et al.*, 2001; Sullivan *et al.*, 2005). Furthermore, HLA-DQA1 * 01 and HLA-DR4 haplotypes of the class II MHC (Major Histocompatibility Complex) appear to be risk factors for the development of CFS/ME (Smith, *et al.*, 2005).

Intestinal dysbiosis hypothesis. 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 (Frissora and Koch, 2005; Riedl *et al.*, 2008). Indeed, the current hypothesis is that in CFS/ME patients some bacterial species are substituted by others able to bind to the same sites of adhesion and induce signals that alter the pre-existing equilibrium. The co-morbidity of CFS/ME and gastrointestinal symptoms has been well proved, with one study reporting that 92% of CFS/ME patients also exhibited IBS (Aaron *et al.*, 2000). Consistently, previous studies have shown an altered composition and reduced biodiversity of oral and intestinal microbiota in CFS/ME patients.

A higher relative abundance of *Leptotrichia*, *Prevotella* and *Fusobacterium* genera and a decrease in the abundance of *Haemophilus*, *Veillonella* and *Porphyromonas* genera were observed in the oral microbiota of CFS/ME patients compared to healthy controls (Wang *et al.*, 2018). *Sheedy* and *colleagues* (2009) found a significant increase in D-lactic acid producing *Enterococcus* and *Streptococcus* species in CFS/ME patients. An association between the increase of *Enterococcus* species, D-lactic acid production and the severity of neurological and cognitive deficits was found in subjects with lactic acidosis (Stolberg *et al.*, 1982). A study conducted on subjects with CFS/ME from Belgium and Norway showed a different composition of the intestinal microbiota between Norwegian CFS/ME patients and healthy controls: in particular, subjects with CFS/ME showed a significant increase in *Lactonifactor* and *Alistipes* genera, paralleled by in the decline of genera belonging to the Firmicutes phylum (*Roseburia, Dialister, Holdemania, Synthophococcus*) (Fremont *et al.*, 2013). A correlation between the symptoms of post-exertion malaise in subjects with CFS/ME and bacterial translocation from the intestine to the systemic circulation was found analyzing the bacterial composition of blood and fecal samples before and after physical exercise. CFS/ME patients showed an increase in the relative abundance of *Firmicutes* and a reduction of *Bacteroidetes* in the bloodstream, while in fecal samples the situation was reversed (Shukla *et al.*, 2015). *Giloteaux* and *colleagues* (2016) observed high levels of some markers for microbial translocation (LPS, LBP and sCD14) in the bloodstream of subjects with CFS/ME and reduced biodiversity of intestinal microbial communities. Recently, an increase in the abundance of *Alistipes* and a reduction of *Faecalibacterium* were observed in individuals with CFS/ME and IBS; instead, a decrease of *Bacteroides vulgatus* and a higher abundance of unclassified *Bacteroides* were observed in CFS/ME patients without IBS (Nagy-Szakal *et al.*, 2017).

It has been suggested that intestinal dysbiosis promotes or is associated with an altered permeability of the intestinal epithelial barrier. The destruction of the junctional complexes in the intestinal epithelium would provide a gateway for the translocation of bacteria (or some of their components) into mesenteric lymph nodes or systemic circulation (Shen *et al.*, 2011; Hietbrink *et al.*, 2009). This can lead to a local and systemic activation of immune cells,

inducing the production of pro-inflammatory mediators and cytokines that can further damage the intestinal epithelium (Turner, 2009). Experimental studies have shown that chronic intestinal inflammation caused by bacterial products can lead to a neurological disease; the lipopolysaccharide (LPS) of bacterial derivation would play a central role in the generation of reactive antibodies against the host's lipoproteins [83] (Vogel *et al.*, 2013).

1.1.3 Diagnosis and therapy

In Italy the CFS/ME is poorly known and diagnosed. Most problems related to CFS/ME diagnosis depend on the lack of specific biomarkers and diagnostic tests, due to the great heterogeneity of CFS/ME populations. Indeed, the diagnosis is based on several criteria, such as the Oxford (Sharpe *et al.*, 1991), Fukuda (Fukuda *et al.*, 1994), and International Consensus Criteria (ICC) classifications (Carruthers *et al.*, 2011), that identify the main symptoms of CFS/ME excluding other clinical conditions that present the same symptomatology (Table 1). Particularly, the main feature for CFS/ME diagnosis is a persistent fatigue that lasts for not less than 6 months, accompanied by at least 4 of the following 8 minor symptoms: severe memory and concentration disorders, pharyngitis, pains in the cervical lymph and axillary nodes, muscle pain, joint pain without swelling or redness, headache, non-restorative sleep, post-effort discomfort lasting more than 24 hours.

TABLE 1 - EXCLUSION CLINICAL CRITERIA FOR THE CHRONIC FATIGUE SYNDROME

- **Oncological diseases**
- **Autoimmune / rheumatological diseases**
- **Infections:** local, occult or systemic, subacute, chronic from bacteria, fungi, parasites, viruses (including HIV and chronic hepatitis)
- **Psychiatric disorders:** bipolar depression, schizophrenia, dementia, anorexia or nervous bulimia
- **Chronic inflammatory disorders** (eg. granulomatosis of Wegener, sarcoidosis)
- **Neuromuscular diseases** (eg myasthenia gravis, sclerosis multiple)
- **Endocrine disorders:** hypothyroidism, d. of Addison, d. of Cushing, IDDM
- **Drug addiction** (alcohol, drugs, psychotropic drugs)
- **Chronic diseases:** cardiac, gastrointestinal, hepatic, renal or haematological

The Oxford criteria consist of two major criteria and eleven minor criteria (symptoms and objective signs). The record of both the major criteria and at least eight out of the eleven minor criteria is necessary to CFS/ME diagnosis. The objective criteria consist of: fever recorded by a doctor, non-exudative, modest pharyngitis, latero-cervical or axillary lymphadenopathy (Sharpe *et al.*, 1991).

According to Fukuda, the first major criterion is the onset of debilitating, persistent or recurrent fatigue which does not resolve resting in bed, reduces the daily patient's activities of at least 50%, and lasts for at least six months. The second major criterion requires the exclusion of

other clinical conditions that could produce the same symptomatology. Following Fukuda's classification, objective criteria are not necessary for diagnosing CFS/ME (Fukuda *et al.*, 1994). Minor criteria include: low-grade fever or chills, pharyngodynia, laterocervical or axillary lymphadenopathy, generalized myasthenia, myalgia, arthralgias migrants without arthritis, headaches, disorders of sleep, generalized prolonged fatigue after exercise, neuropsychological disorders (photophobia, amnesia, irritability, mental confusion, concentration difficulty).

Currently, there is no effective therapy for CFS/ME. Both pharmacological and non-pharmacological (cognitive-behavioral and rehabilitative) therapies are usually applied. Non-pharmacological therapy is based on the emotional support of the patient, reduction of symptoms and improvement in the quality of life. Drug therapy involves the use of psychotropic (sedatives, anxiolytics, antidepressants), immunomodulating (immunoglobulins) and cortisone drugs, non-steroidal anti-inflammatory drugs (NSAIDs) and antivirals.

In recent years, attempts have been made to treat the disease by probiotic oral and rectal administration. Improvements in the majority of CFS/ME patients following bacteriotherapy by rectal infusion of enteric bacteria were reported in a study carried out on subjects with fibromyalgia, IBS and CFS/ME (Borody *et al.*, 2012). In two other studies, slight improvement in certain symptoms was observed in patients following oral probiotic therapy (Sullivan *et al.*, 2009; Rao *et al.*, 2009).

1.2. The human microbiota

The human microbiota is the set of microbial populations constituted mainly by commensal, symbiotic and pathogenic bacteria and other micro-organisms - Archaea, Eukarya, fungi and viruses - that colonize humans at birth and from which the host organism depends for various functions (Nicholson *et al.*, 2012; Kau *et al.*, 2011).

The microbiota is transferred from mother to child at birth and affects the life and health of the child with epigenetic effects. Recent evidence suggests that the microbial colonization of the gut starts before birth, as revealed by the placental microbiome profile (Aagaard *et al.*, 2014). The infants born with natural birth have a microbiota containing species derived from the vaginal microbiota of their mothers. Conversely, in the case of cesarean section, the microbiota is similar to the skin microbiota, with many environmental bacteria that colonize the newborn together with those of maternal origin (Dominguez- Bello *et al*., 2010).

Birth mode seems to influence immunological maturation through the development of the microbiota. Children born by cesarean section were found to have a higher number of antibody-secreting cells (Clemente *et al.*, 2012). Moreover, also human milk is involved in the development of both the intestinal microbiota and immune system: milk contains several bioactive substances and immunological components that control the maturation of the newborn intestine and the composition of the microbial community. Breastfeeding has a protective role in infants, provided by a complex mixture of molecules (lysozyme, IgA, alpha-lactalbumin, lactoferrin, free oligosaccharides, complex lipids) (Gordon *et al.*, 2012).

The stabilization of the microbiota is achieved around 3 years of age (Dominguez- Bello *et al.*, 2010). During the course of life the microbial communities increase linearly their diversity in relation to several modulating factors (age, diet, lifestyle, geographical location, genotype, drug intake) (Thursby and Juge, 2017). A total of 10-100 billion microbial cells dwell into the whole human body, except the circulatory system and the brain, for a bacterial/human cells ratio of 10:1 (Ursell *et al.*, 2013). The human genetic materials and those of its microbiota form the human metagenome. The totality of the genes that the microbiota is able to express is called microbiome, with a bacterial/human genes ratio of 1000:1 (Qin *et al.*, 2010). The human metagenome constitutes a dynamic entity, conditioned by the diversity of microbial species in symbiosis with humans. The host genome determines the availability of adhesion sites for microorganisms in different parts of the body; indeed, new species of bacteria are always available and ready to bind previously occupied sites which have become available. In its turn, the level of expression of many host's genes depend on the microbial composition, which affects the levels of proteins and enzymes in the host.

Given the importance of this symbiosis and its implications for human health, the Human Microbiome Project was started in 2008 by the National Institutes of Health (NIH) whose purpose is the cataloging of the human microbiome and the analysis of its role in health and disease (*hmpdacc.org/*). The human microbiota harbors in several body districts: the intestine, oral cavity, nasopharyngeal tract, skin and urogenital tract (Fig. 2).

Eubacteria are the most abundant group and are represented by about 1000 species, of which less than 200 species live in each individual (HMP Consortium, 2012). The oral and gut microbiota are the most complex microbial community in the human body and are closely connected (Rautava *et al.*, 2015).

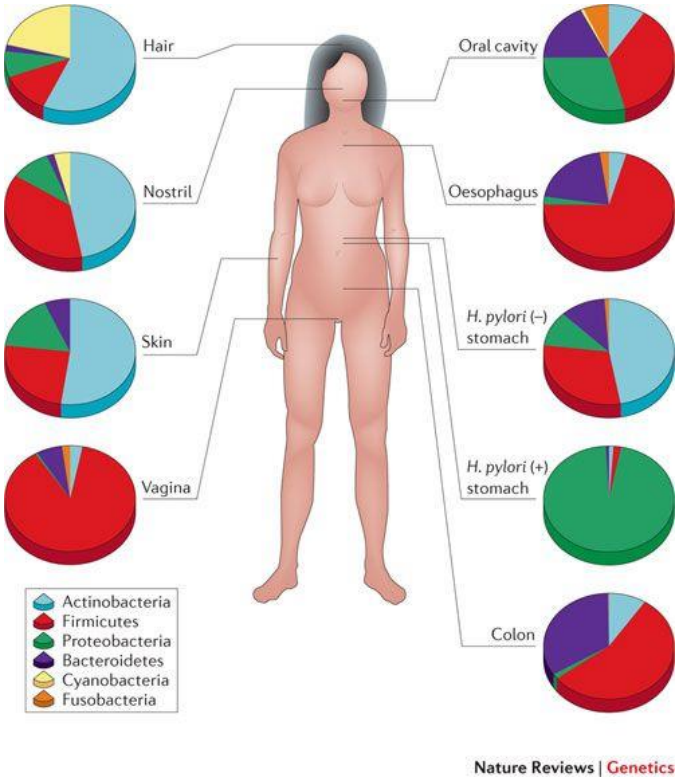

Figure 2. Human microbiota composition. *The figure illustrates the distribution of bacterial communities in different body sites. (Imagine retrieved from Cho I. and Blaser MJ, Nature Reviews Genetics, 2012)*

The oral cavity represents the gateway for bacteria at the first contact of the newborn with the external environment and then the colonization involves the gastrointestinal tract. Oral microorganisms form one of the most diverse microbiota in the human body and include bacteria, archaea, fungi, viruses and protozoa. The oral microbiota is directly in contact with the external environment and subjected to modifications that affect the composition of the bacterial flora in the other body sites. The Human Oral Microbiome Database (HOMD,

www.homd.org) catalogs and shares information on the microorganisms of human oral cavity. Bacteria are predominant and about 1000 species are found in the mouth. Particularly, the most representative phyla are: *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *TM7* and *Spirochaetes* (Wade, 2013). The oral bacterial communities include both obligate aerobes and facultative and obligate anaerobes, able to degrade sugars and proteins, and several complex substrates derived from them. Many oral bacteria are facultative anaerobes, the two most numerous genera being *Actinomyces* and *Streptococcus*, while obligate aerobes are few, mostly *Rothia* and *Neisseria* species (Diaz *et al.*, 2006).

Interestingly, the composition of the oral microbiota does not vary among populations that live in different geographic areas (Nasidze *et al.*, 2009).

The intestinal microbiota is the most complex symbiotic community, including 70% of all the microorganisms dwelling in the human body. The composition of the intestinal microbiota changes through the digestive tract (Table 2).

Table 2. Main habitats of the human gastrointestinal tract and resident bacteria.

Districts	**Resident bacteria**	**Abundance**
Stomach	*Helicobacter pylori, Steprococcus, Lactobacillus.*	10^2 - 10^3 CFU/mL
Small intestine	*Bacteriodes, Ruminococcus, Clostridium, Caprococcus, Streptococcus, Lactobacillus*	10^2 - 10^9 CFU/mL
Large intestine	*Enterobacteria, Enterococcus faecalis, Escherichia Coli, Bacteroides, Bifidobacterium, Eubacterium, Peptostreptococcus, Ruminococcus, Clostridium, Lactobacillus*	10^9 - 10^{14} CFU/mL

Particularly, the colon is the most densely populated tract including 10^{14} bacterial cells belonging to between 300 and 1000 different species (Thursby and Juge, 2017). The dominant phyla in the human gut are *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*.

Most bacteria belong to the genera *Eubacterium*, *Bacteroides*, *Faecalibacterium*, *Clostridium*, *Ruminococcus*, *Bifidobacterium* and *Peptostreptococcus*, while other genera, such as *Lactobacillus* and *Escherichia*, are present to a lesser extent. Species belonging to the genus *Bacteroides* represent about 30% of all gut bacteria (Thursby and Juge, 2017). The microbiota

varies between individuals in relation to several factors. Among these, the diet is certainly the most important modulating factor of intestinal bacterial diversity, influencing its functional relationships with the host (Arumugam *et al.*, 2011).

The intestinal microbiota can be divided into three large groups, called enterotypes (Arumugam *et al.*, 2011), based on the greater relative abundance of one of three bacterial genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), *Ruminococcus* (enterotype 3) (Fig. 3). The different composition of the microbiota assimilable to enterotypes is observed inside the colon. An individual's enterotype does not depend on nationality, sex, age or body weight, but exclusively on diet. *Bacteroides* and *Ruminococcus* enterotypes are associated with either lipid- or protein-rich diets, while *Prevotella* with carbohydrates. Indeed, the western, meat-based lifestyle is associated with enterotypes 1 and 3, while vegetarian/vegan, cereal-based diets are associated with enterotype 2 (Gorvitovskaia *et al.*, 2016).

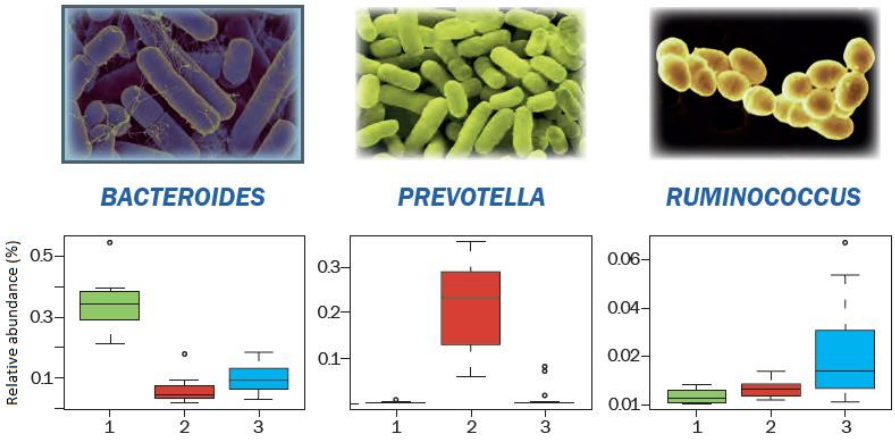

Figure 3. Enterotypes in the human gut. *(Imagine retrieved from https://www.ibsafoundation.org/wp-content/uploads/2018/06/8-il-microbiota-lebba.pdf)*

1.2.1 Factors that modulate the intestinal microbiota composition

As mentioned above, there are several factors that play an important role in the development and modeling of a normal intestinal microbiota. Diet is certainly one of the main factors modulating the composition of the microflora, together with age, sex, ethnicity, genotype, environmental conditions and the use of antibiotics (Thursby and Juge, 2017).

A study conducted on African and European children recorded significant differences in intestinal microbial composition between European children and those of an African rural village in Burkina Faso. African children showed higher prevalence of *Bacteroidetes* and *Prevotella* species, known to possess a number of bacterial genes for the digestion of cellulose,

while in European children *Firmicutes* and *Bacteroides* prevailed. These differences are related to the diet of the two populations: European children's diet was rich in animal protein, sugars, starch, fat and poor in fibers, while African children ate foods rich in fibers (De Filippo *et al.*, 2010).

Many studies have shown the existence of sex-related differences regarding the composition and function of the intestinal microbiota. These variations, partly driven by sex hormones, lead to differences between the two sexes that relate to immunity and susceptibility to various diseases (Vemuri *et al.*, 2019).

Even antibiotics are able to alter the composition of the intestinal microbiota, inducing positive effects, such as increased abundance of beneficial bacteria. However, a growing evidence suggests that the excessive use of antibiotics can lead to the development of diseases caused by changes in the intestinal microbiota and the development of antibiotic resistance (Ianiro *et al*., 2016).

1.2.2 Role of microbiota in health

The term "eubiosis" indicates a state of balance in the intestinal microbial ecosystem. In a state of eubiosis, the intestinal microbiota is characterized by beneficial species, mainly belonging to the phyla *Firmicutes* and *Bacteroidetes*, while potentially pathogenic species, such as the *Proteobacteria* (*Enterobacteriaceae*) are present in a very low percentage (Zhang *et al.*, 2015). The beneficial bacteria protect the intestinal mucosa, facilitating the digestive and assimilative processes. The balance between the various groups of these bacteria is essential for health.

Oral health has a great impact on host health and is strongly influenced by oral microbiota, which, in its turn, plays a major role in metabolism, nutrient digestion and immune response (Moye *et al.*, 2014; Slocum *et al.*, 2016). The most important functions of oral microbiota are nutritional. The primary substrates for bacterial growth in the mouth are derived from saliva in the form of glycoproteins rather than from the food. *Streptococcus* species have both glycosidic and endopeptidase activity and are both able to degrade oral glycoproteins (Byers *et al.*, 1999; Homer *et al.*, 1990). Members of the genera *Porphyromonas* and *Prevotella* also have endopeptidase activity, resulting in the cleavage of proteins to peptides (Bao, *et al.*, 2008). *Fusobacterium* and *Peptostreptococcus* exhibit aminopeptidase activity and can ferment amino acids producing short-chain fatty acids (Ng *et al.*, 1998; Rogers *et al*., 1998).

In a status of equilibrium, which can be identified with healthy conditions, the gut microbiota is involved in the host's metabolism, immunomodulation, pathogen resistance, maintenance of intestine structure/function and nutrition (Nicholson *et al.*, 2012; Kau *et al.*, 2011).

The intestinal microbiota is particularly involved in the degradation of complex carbohydrates (Rowland *et al.*, 2018). The intestine acts as a bioreactor for carbohydrates (Fig. 4). Humans absorb simple sugars in the small intestine, but their ability to digest polysaccharides is low. Undigested polysaccharides (i.e. cellulose, xylan, undigested starch or endogenous glycans such as mucins and glycosphingolipids) pass through the distal parts of the small intestine (ileum) and the colon and are degraded by the microorganisms of the intestinal microbiota. Many intestinal bacteria have a high number of enzymes (i.e glycoside hydrolases, polisaccharide lyases, carbohydrate esterases) which allow them to use undigested carbohydrates as food.

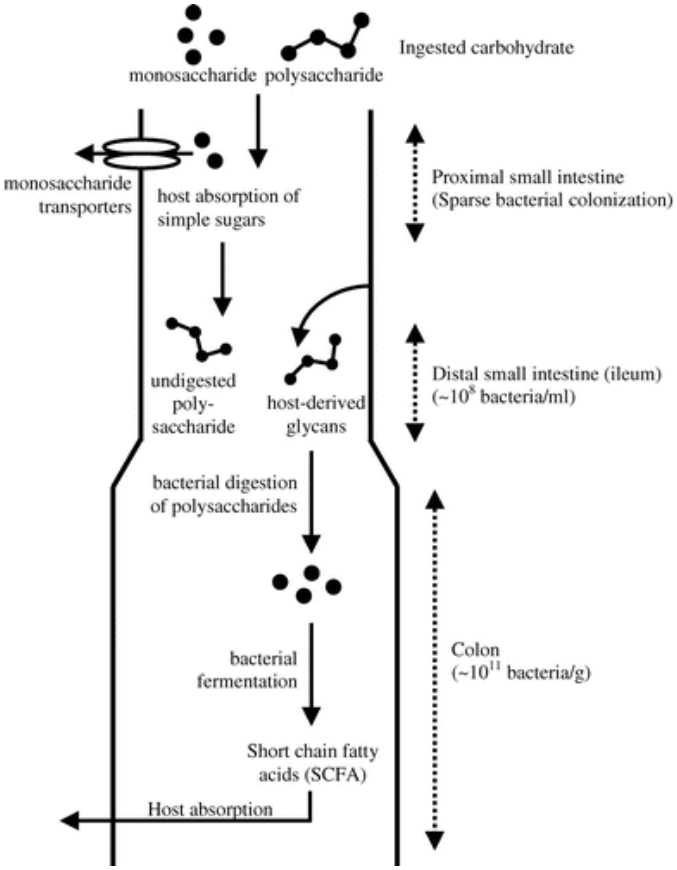

Figure 4. Representation of host and bacterial contributions to carbohydrate utilization in the intestine. *(Imagine retrieved from Hooper L.V. et al, Annu. Rev. Nutr., 2002)*

The result of this degradation is the formation of monosaccharides which are then converted into bacterial fermentation products such as short-chain fatty acids (SCFAs: acetic, lactic, butyric, propionic acids), which are a primary energy source for enterocytes (Lora *et al.*, 2002).

Released monosaccharides and produced SCFAs can then be absorbed and used by the host. The bacterial production of SCFAs is involved in the regulation of intestinal trophism, promoting the proliferation and differentiation of the intestinal epithelium, and play an important anti-inflammatory role.

The microbiota also contributes to the synthesis of B vitamins (in particular, B12) and growth factors (Rowland *et al.*, 2018), metabolism of lipids and proteins and degradation of various polyphenols introduced through the consumption of fruit and products derived from plants (Jandhyala *et al.*, 2015). Moreover, the microbiota is also able to metabolize xenobiotics and drugs (Jandhyala *et al.*, 2015).

Intestinal bacteria play an important role in the maturation and regulation of the immune system. Through a mechanism called immunomodulation, the microbiota helps the development and maturation of GALT (the lymphoid tissue associated to the intestine) and the organization of Peyer plaques and isolated lymphoid follicles (Jandhyala *et al.*, 2015). Commensal microorganisms are necessary for the maturation of the immune system, which learns to distinguish commensal bacteria from pathogens through the toll-like receptors (TLR) of the epithelial and lymphoid cells of the small intestine (Lazar *et al.*, 2018).

TLRs repress the occurrence of inflammatory processes and promote immunological tolerance to normal microbiota components. They recognize different general microbe-associated molecular patterns (MAMPs) and activate the innate intestinal immunity (Francino, 2014). After that, a complex cascade of signals is started, causing the release of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), which activates several genes coding for chemokines, cytokines, acute phase proteins, and other effectors of the humoral immune response (Belkaid and Hand, 2014; Thomas and Versalovic, 2010).

Soon after birth and throughout life, the intestinal microbiota stimulates the development of the immune system and acts as a barrier against the proliferation of pathogens by inhibiting the pathogens' adherence and colonization and the production of bacteriocins and other toxic metabolites (mechanism known as "colonization resistance" or "competitive exclusion") (Kamada *et al.*, 2013).

The intestinal microbiota is involved in the regulation of the Central Nervous System (CNS) through the microbiota-gut-brain axis (Zhul *et al.*, 2017), a bi-directional communication between the microbiota, the Central Nervous System (CNS), the autonomic and enteric nervous system and the hypothalamic-pituitary-adrenal (HPA) axis through neurological, endrocrine and immunological pathways (Fig. 5). The intestinal microbiota synthesizes a large number of chemical compounds which can reach distant sites, such as the brain, through the blood stream, exerting either positive or negative effects on the host health (Clarke *et al.*, 2014). Indeed, the

intestinal bacteria are able to produce several metabolites, including neurotransmitters such as dopamine, serotonin and GABA, which play an important role in the chemical signaling between the microbiota itself and the brain (Clarke *et al.*, 2014). Therefore, alterations in the composition and abundance of intestinal microorganisms can influence both the enteric nervous system and the CNS, affecting cognitive and behavioral functions (Zhul *et al.*, 2017).

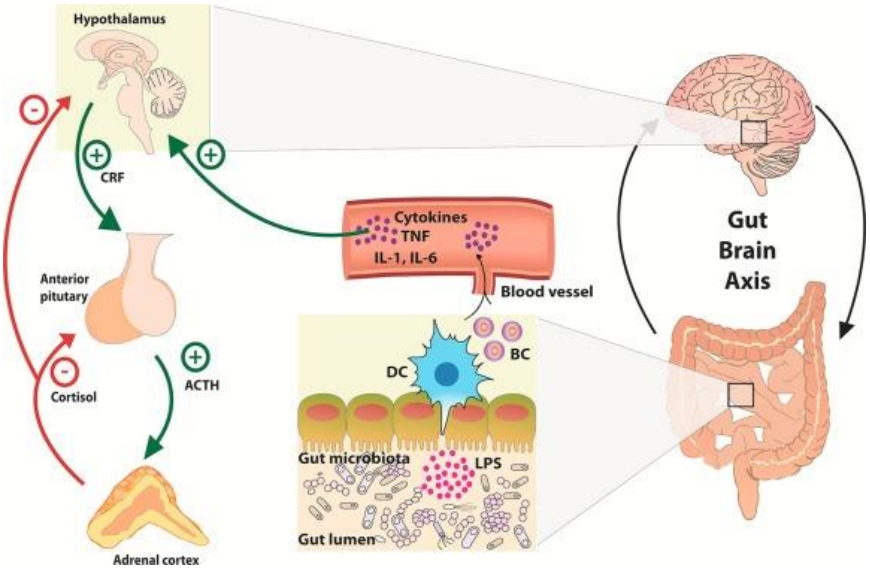

Figure 5. Microbiota-gut-brain axis. *The gut microbiota regulates the release of cytokines TNFα, IL-1 and IL-6, by the cells of the enteric immune system into the bloodstream, which reach the brain and stimulate the release of CRF from the hypothalamus. CRF actives the anterior pitutary and the release of ACTH that induces the adrenal cortex to release cortisol. Abbreviations: CRF corticotrophin-releasing factor, ACTH adrenocorticotropic hormone, LPS lipopolysaccharide, DC dendritic cell, BC B cell, IL interleukin, TNF tumor necrosis factor α. (Imagine retrieved from Cussotto S. et al, Frontiers in Neuroendocrinology, 2018)*

1.2.3 Role of microbiota in disease

Recently, several studies have pointed out the important role played by the microbiota in host health and, conversely, its contribution to disease development (Robinson *et al.*, 2010; Bassis *et al.*, 2013).

An alteration of the microbial composition, in terms of changes in species numbers and their prevalence, causes the loss of homeostasis, a condition called “dysbiosis”, which can lead to the dominance of pathogenic species and increase in harmful compounds. In dysbiosis condition, the genetic coding of useful molecules is lost and harmful metabolites are produced by pathogenic microorganisms, affecting the pathogenesis of diseases and causing intestinal inflammation.

Changes in intestinal microbial composition may be due to various factors such as infections by pathogenic bacteria, viruses or fungi, excessive use of drugs and antibiotics and also different conditions of stress (Karl *et al*., 2018). The effects of stress include the increase of intestinal

permeability and alteration of motility and gastrointestinal secretions. Through these physiological mechanisms, stress can alter the microbiota, inducing the release of stress hormones by microbial communities which can enhance the growth of specific pathogens and their ability to adhere to the intestinal mucosa (Diaz *et al.*, 2011).

Some bacterial species that colonize the oral cavity are potentially pathogenic. The oral microbiota is responsible for the three commonest oral diseases in humans, periodontitis (Darveau, 2010), gingivitis (Zijnge *et al.*, 2010), tooth decay (Kaur *et al.*, 2013). Several studies have described the association of oral microbiota also with non-oral diseases such as respiratory infection (Raghavendran *et al.*, 2007), pancreatic (Cavestro *et al.*, 2016) and intestinal diseases (Kuehbacher, 2008). Changes in the intestinal microbial composition have been detected in metabolic diseases such as type 2 diabetes (Qin *et al.*, 2012) and obesity (Ridaura *et al.*, 2013), IBS (Labus *et al.*, 2017; Tap *et al.*, 2017), IBD (Manichanh *et al.*, 2006; Maukonen *et al.*, 2015), autoimmune conditions (Marasco *et al.*, 2016; Hevia *et al*., 2014), cancer (Wu *et al*., 2009) and in several neurological disorders such as Parkinson's disease (Scheperjans *et al.*, 2015), Alzheimer's disease (Zhuang *et al.*, 2018) and autism (Finegold *et al.*, 2010), highlighting that there is a strong correlation between dysbiosis and the pathological condition.

Alterations in the prevalence of several genera belonging to *Lachnospiraceae* family (*Firmicutes*) and an increased prevalence of *Bacteroides* have been described in Chron's disease (Manichanh *et al.*, 2006), ulcerative colitis (Maukonen *et al*., 2015) and Systemic Lupus Erythematous (Hevia *et al*., 2014). Culture-based analysis revealed that the levels of *Fusobacterium* and *Porphyromonas* were significantly higher in subjects with oral squamous cell carcinoma compared to healthy controls (Nagy *et al.*, 1998).

Knowledge of microbiota composition can be a key factor for the identification of a disease and choice of the most appropriate treatment. Currently, therapeutic measures and protocols based on the manipulation of intestinal microbial composition are used for the treatment of several pathological conditions. In recent years, the use of probiotics, prebiotics and fecal microbiota transplantation has become increasingly popular (Bergmann *et al.*, 2014; Leshem *et al.*, 2019).

Probiotics are defined by The World Health Organization as living microorganisms which are able to confer benefits to the host if taken in the correct quantity. They mimic normal physiological conditions, enhancing the development the beneficial bacteria and hence promoting health by reinforcing the intestinal barrier, stimulating immunity, producing vitamins and antioxidants and controlling the overgrowth of pathogens (Santocchi *et al.*, 2016). The most used probiotics are *Bifidobacterium* and *Lactobacillus* species (Fond *et al.*, 2015).

Prebiotics occur in food characterized by the presence of non-digestible oligosaccharides, such as galactooligosaccharides (Bergmann *et al.*, 2014). They induce specific changes in the

composition of gastrointestinal microbiota, promoting the growth and activity of beneficial bacteria.

In recent years, the transplantation of fecal microbiota is gaining increasing interest for its potential use in humans as a therapeutic measure against diseases associated with intestinal dysbiosis. This strategy has been applied for the treatment of colitis caused by *Clostridium difficile*, but some studies show that it can also be used for the treatment of obesity, diabetes, depression and chronic inflammatory diseases (Bakker and Niewdorp, 2017; De Groot *et al.*, 2017).

The main advantage of fecal microbiota transplantation is to reduce the risk of inducing antibiotic resistance in highly pathogenic bacteria. In addiction, this strategy is relatively inexpensive, being a non-pharmacological medical treatment. However, fecal transplantation represents an invasive method, compared to conventional treatment with antibiotics, and a risky strategy due to the possible transmission of infections (bacteria, viruses, intestinal parasites). Evidence relating to the safety of fecal transplantation is relatively limited. This is because the inclusion of this method in clinical practice took place very quickly and before large long-term prospective studies had been carried out, normally foreseen to evaluate the safety of new interventions. In general, fecal transplantation has been shown to be safe and effective in the short term, while data on long-term safety are still limited and it is not yet clear how the introduction of the microbiota affects the physiology of the host in the long term (Kelly CR et al., 2015). In recent years, several research teams have been trying to find the "ideal" fecal sample for the treatment of certain pathologies such as the immune-mediated ones, thanks to the strong link between immunity and microbiota.

The administration of probiotics and prebiotics and the use of fecal transplantation for the treatment of diseases aim to enrich the intestinal microbial ecosystem with beneficial bacteria, which play a key role in human health, and to restore the equilibrium status.

1.3 Methods for the characterization of microbiota by sequencing

To date, the study of microorganisms has been carried out mainly according to a culture-dependent approach that is through the cultivation of microorganisms in culture media, mainly based on the morphological identification of microbial colonies and functional analysis by biochemical assays. However, the application of traditional microbiological techniques does not allow the identification of all the microorganisms really dwelling in a certain habitat, because the majority of microbial species (96-99%) cannot be cultivated in the laboratory (Hugenholtz *et al.*, 1998).

Recently, new molecular technologies have provided the basis for a more efficient microbial identification.

1.3.1 Metagenomic analyses

Metagenomics is a branch of genomics that studies a complex community of microorganisms directly accessing to their genetic contents and avoiding growth on selective culture media (culture-independent method). The overcoming of classical microbiology techniques for the identification of microorganisms involves the use of total genomic DNAs extracted from various matrices (soil, wood, oil, milk, ocean sediments, water sampled in different parts of a river's course, agricultural silage, stone fragments taken from monuments) and the sequencing of genomic regions, amplified by "universal" primers the target of which are gene sequences conserved among the organisms of interest. Most used regions are those encoding the hypervariable regions of the 16S rRNA gene. In this way, it is possible to depict to a larger extent the diversity of bacterial communities occurring in different habitats. Moreover, metagenomic analysis allows to study how the various microorganisms interact with each other and the environment and determine the specific functions of a microbial community.

1.3.2 16S rRNA gene

The 16S rRNA gene is ca. 1540 bp long and consists of 10 conserved regions and 9 hypervariable regions (V1-V9) (Fig. 6). The product of 16S rRNA is the minor subunit 30S of prokaryotic ribosomes (Srinivasan *et al.*, 2015).

Occurring in all bacteria and archaea, the 16S rRNA gene is widely used as a phylogenetic marker to examine microbial diversity and identify and classify microorganisms. It is not subject to lateral gene transfer, and its hypervariable regions allow the discrimination of

different bacterial species. Moreover, the presence of several highly standardized and updated 16S databases allows the prompt identification of bacterial species.

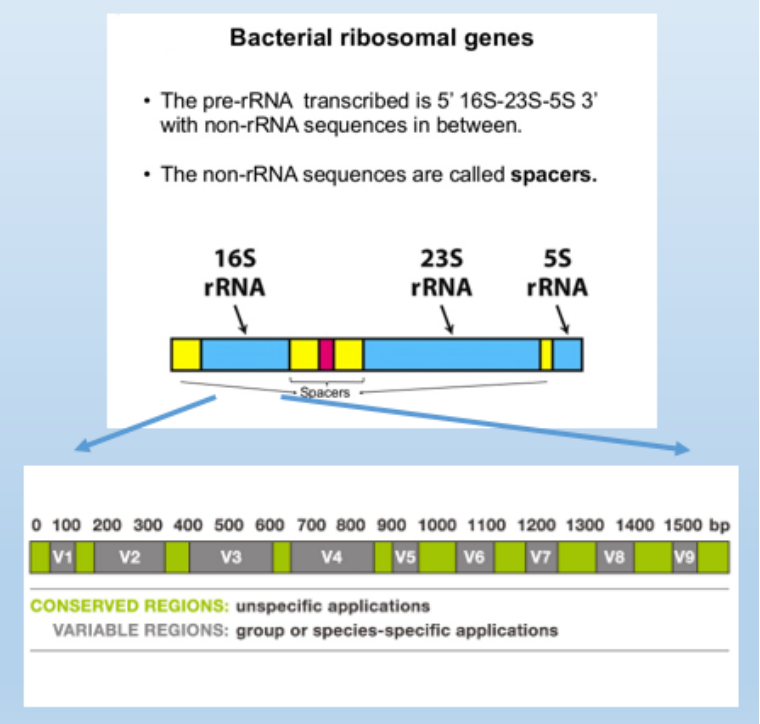

Figure 6. 16S rRNA gene. *Subdivision of hypervariables regions within 16S rRNA gene.*

NGS analysis is carried out selectively on some hypervariable regions. Indeed, some of the nine hypervariable regions, and in particular V1-V2 and V3-V4 regions, are more informative than others (Yu and Morrison, 2004) and allow a more reliable taxonomic assignment than others (Liu, et al, 2008).

Several NGS platforms are used for the global analysis of abundance and diversity of complex microbiomes (i.e pyrosequencing Roche-454, Illumina, SOLiD). The individual hypervariable sequenced regions are grouped into "Operational Taxonimic Units" (OTUs), i.e. taxonomic clusters of sequences based on sequence similarity. Taxonomic identification is based on the sequencing of the nine hypervariable regions of the 16S rRNA gene.

1.3.3 First generation sequencing methods

Sanger's method, also known as chain termination method, is the first enzymatic sequencing technique proposed to identify the amino acid sequences of a DNA fragment (Sanger *et al.*, 1977).

This technique is based on the use of modified nucleotides (ddNTP), characterized by the substitution of ribose with dideoxyribose, that prevents binding to other nucleotides. The PCR requires multiple copies of a DNA fragment that acts as template, a DNA polymerase, a primer for DNA synthesis, the four types of deoxynucleotides triphosphates (dATP, dGTP, dCTP and dTTP) and ddNTPs, labeled radioactively or with fluorochromes of different colors. Whenever, occasionally and randomly, ddNTPs are included in the DNA chain, that is forming on the basis of one of the DNA template fragments, the reaction ends irreversibly. The amplification of the DNA template provides a plethora of fragments of different length, each of which ends with a particular labeled ddNTPs. Subsequently, DNA fragments are split according to their length by gel electrophoresis. During the electrophoretic run, the fragments are excited by a laser source, thus allowing the formation of a particular trace. This is analyzed by a specific software, which translates it into a sequence of nucleotides. The type of base is identified based on the color of the fluorochromes recorded during the electrophoretic run. The position of the bases is reconstructed from the order of the fragments at the end of the electrophoretic run. The advantage of this method is the ability to sequence relatively large (> 700 bp) amplicons with low error rates.

The most common technique used among culture-independent methods is the Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis (PCR–DGGE). DGGE of PCR-amplified 16S ribosomal DNA (rDNA) fragments has been frequently applied to the fingerprinting of bacterial populations in many fields, such as soil, sea and clinical samples. (Strathdee and Free, 2013)

This technique involves the DNA extraction from samples, the amplification of 16S rDNA genes with bacterial specific primers, the electrophoresis on acrylamide gel containing a vertical gradient denaturant (urea and formamide).

The DGGE technique is based on the separation of PCR products of the same molecular weight but with a different nucleotide sequence. This separation of double-stranded DNA (dsDNA) fragments takes place based on the principle that dsDNA molecules, subjected to a gradient of chemical denaturing agents (urea and formamide), undergo denaturation and therefore a change in conformation that slows down the migration speed on the gel. Particularly, during electrophoresis, the DNA fragment remains double-stranded until it reaches the area of the gel corresponding to its melting temperature (Tm). At this point, the fragment is partially denatured and therefore has a reduced electrophoretic mobility. The complete dissociation of the dsDNA is prevented by the addition to the 5 'end of the forward primer of a tail formed by about 40 bases of G + C.

All PCR products, loaded on DGGE gel, dissociate in different points of the denaturing gradient and are distinguishable based on the different electrophoretic migration, thus determining a microbial composition profile. After gel staining, the number of bands is indicative of the number of microbial species present in the initial sample, providing an estimate of microbial diversity in a given ecosystem. Finally, it is possible to obtain taxonomic information since the bands can be excised from the gel, re-amplified and sequenced.

However, DGGE has numerous disadvantages: variable efficiency of metagenomic DNA extraction; amplification errors and heteroduplex formation during the PCR reaction. In addition to this, only small fragments (<500 bp) can be separated in DGGE and this strongly limits the sequence information.

In the last years, Sanger's and DGGE methods have been gradually replaced by new generation technologies.

1.3.4 Next Generation Sequencing (NGS) platforms

NGS techniques are based on sequencing by synthesis and requires the amplification of DNA fragments in clusters that are subsequently anchored on microplates and introduced into a flow cell where sequencing reactions take place. A primer is extended cyclically by adding one or more nucleotides during the amplification and the sequence is read at each step of DNA synthesis.

Currently available methods differ in the strategy used for amplifying the sequences, in the chemistry used and in the length of reads. Major benefits of NGS platforms are 1) the possibility of sequencing up to several million DNA fragments in parallel, 2) elimination of the cloning phases prior to sequencing, now replaced by a ligation of specific adapters and subsequent amplification, 3) increase of sequencing speed and 4) low costs for the production of DNA sequences.

Below, two NGS technologies are described: Roche-454 and Illumina sequencing.

Roche-454 pyrosequencing. Pyrosequencing requires the preparation of a DNA library: DNA is denatured and reduced to fragments to which adapters are added by ligation; successively, each fragment is anchored on the surface of beads, which are incorporated in water-in-oil emulsions. Amplicons are generated by emulsion PCR and, finally, pyrosequencing is performed. This technique is based on the detection of inorganic pyrophosphate (PPi), obtained by inserting a nucleotide into the growing chain during DNA synthesis. After insertion, PPi is

converted to ATP by sulfurylase. ATP is then used by luciferase to convert luciferin into oxyluceferin, producing a light signal that can be detected by a CCD camera.

Illumina. Also the Illumina technology involves the preparation of a DNA library but, unlike pyrosequencing, amplification takes place through the PCR bridge. Two types of primers, forward and reverse, are anchored on a rigid support. The amplified filament is folded in the direction of the nearest primers on the support and is aligned with one of them; therefore, a further amplification occurs in the opposite direction. This step is repeated many times for the same DNA chain to obtain a cluster of amplicons folded on themselves. After that, DNA filaments are denatured in order to obtain single-chain and non-folded amplicons. At this point sequencing begins: four types of modified nucleotides (ddNTP), each containing a different fluorescent marker, are introduced on the platform. During the replication of amplicons, at every incorporation of a ddNTP in the growing chain the reaction stops. Then, the fluorescence emitted after the ddNTP incorporation is captured by an image acquisition system, which allows to show which base has been incorporated at each cycle. At the end of the acquisition, the support is washed and a new cycle of sequencing can start (Fig. 7).

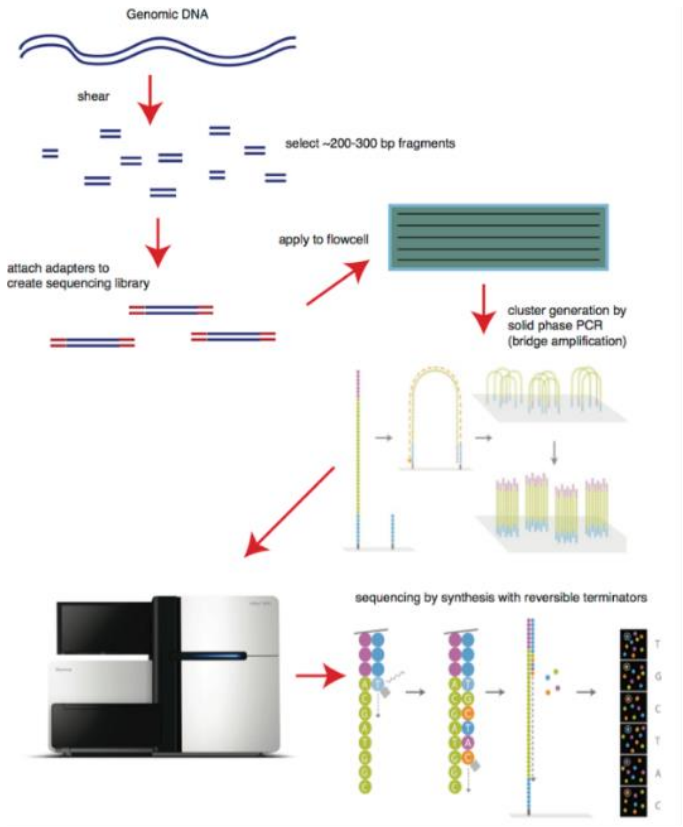

Figure 7. Illumina technology. *The workflow of Illumina sequencing is illustrated. (Imagine retrieved from https://bitesizebio.com/13546/sequencing-by-synthesis-explaining-the-illumina-sequencing-technology/)*

1.4 Metabolomic analysis

The complexity of a biological system is based on dynamic interactions among a large number of cellular components, such as genes, proteins and metabolites.

Metabolomics is the study of the metabolome, defined as the set of metabolites produced by or otherwise occurring in a biological system (Clish, 2015). Metabolomics allows a quantitative and qualitative characterization of all metabolites and to highlight their interactions. As the metabolic profile is strongly influenced by environmental factors (diet, environmental agents, drugs or other substances), metabolomics also allows analyzing the effects of the interactions with external agents (Clish, 2015).

In the clinical field, metabolomic analysis allows the identification of single metabolites or metabolic patterns and represents a useful instrument to understand the etiology of a disease. Furthermore, metabolomics allows the identification of unknown metabolites (Bingol and Bruschweiler, 2017). Pathological phenotypes are identified by pointing out the set of metabolites that discriminates between different groups of subjects. Therefore, these metabolites can represent potential biomarkers of disease (Gebregiworgis and Powers, 2012).

The aim of metabolomics is to study the set of metabolites of a given biological sample, which includes a large variety of different molecules (such as amino acids, carbohydrates, lipids and organic acids). These compounds occur in different concentrations and in several biofluids and tissues (Riekeberg and Powers, 2017).

Different approaches and methodologies are used. *Target analysis* is a specific approach, based on the qualitative and quantitative analysis of a single compound, such as a disease marker. The *profiling metabolite* focuses on the analysis of either a group of metabolites belonging to a same class of compounds or a specific metabolic pathway. *Target analysis* and *metabolic profiling* approaches are based on previous knowledge and on the study of a small number of metabolites (Clish, 2015; Tan *et al.*, 2016).

Converserly, *metabolite fingerprinting* is an approach that allows to examine the largest number of metabolites for the definition of metabolic patterns associated with a given condition. This approach aims to define the metabolic characteristics that are able to discriminate between the groups in study. *Metabolite fingerprinting* is not based on previous knowledge and involves a large number of metabolites.

To date, the exact number of human metabolome components is still unknown.

The main analytical methodologies used in metabolomics are Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry (MS), often coupled with chromatographic separation methods (Clish, 2015).

The data obtained from the analysis are elaborated with dedicated software and processed by multivariate statistical methods that allow to identify the most significant variables for the characterization of a pathological condition. The final step is the chemical-physical identification of the metabolites, which is achieved by comparison with metabolite databases (such as Human Metabolome Database- HMDB).

Applying mass spectrometry, analytes can be ionized in positive or negative and the ions are separated according to their mass/charge ratio (m/z) to be then represented in a spectrum with their intensities. MS analysis is usually coupled with chromatographic separation methods, such as liquid chromatography or gas chromatography, which allow to increase the analytical sensitivity and the number of identified metabolites. M/z ratio, retention time and intensity are the parameters that represent the metabolic fingerprinting of the sample under investigation (Fig. 8).

Multivariate methods applied for processing metabolomic data are classified as *unsupervised* and *supervised* (Boccard, et al., 2010). Applying *unsupervised* methods, the classification of groups is not previously known. These methods aim to evaluate whether the comparison of metabolic spectra allows the discrimination of the different groups under investigation. An *unsupervised* method widely used in metabolomics is Principal Component Analysis (PCA).

With *supervised* methods, the classification of groups is previously known, and the analysis aims to obtain a mathematical model which, based on metabolomic data, allows to identify the variables that discriminate between groups. The resulting model allows to predict which group a possible new sample belongs to.

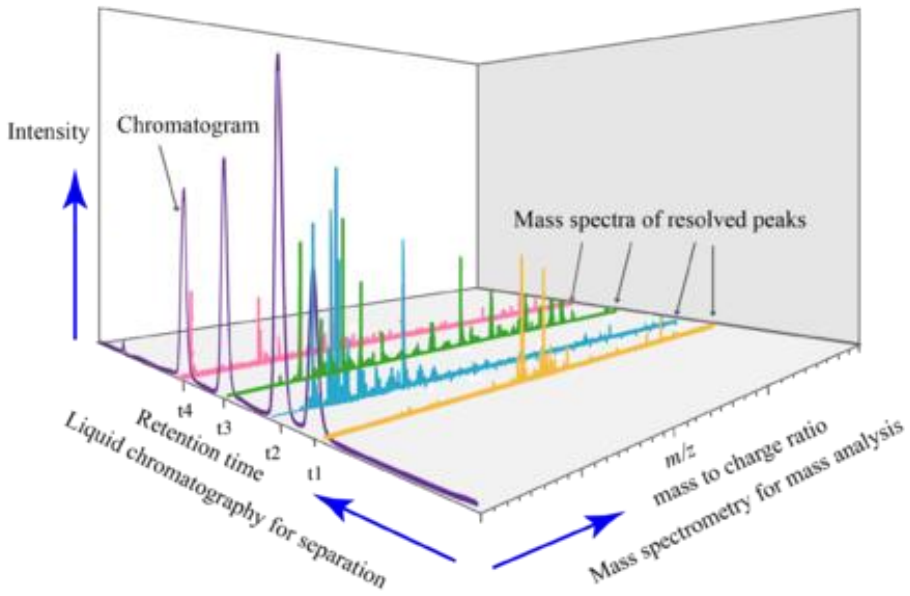

Figure 8. LC-MS Spectrum of each resolved peak. (*Imagine created in Illustrator C6 by Daniel Norena-Caro)*

Online metabolic databases provide information on the chemical-physical and spectroscopic characteristics of known metabolites which can be compared with those of the metabolites to identify. The Human Metabolome Database is the most complete and contains information on over 40000 metabolites (*www.hmdb.ca*).

More sophisticated analytical techniques are required for the accurate identification of the metabolites. As each element has isotopes with a characteristic mass, using high resolution instruments it is possible to measure the mass precisely and thus obtain very precise information on the composition of the examined metabolite. Another method consists in the use of fragmentation methods, such as MS/MS mass spectrometry: an ion, selected by the first analyzer, is fragmented in a collision cell and the mass analysis of its fragments is performed in a second analyzer. Each compound has its own characteristic fragmentation spectrum, so MS/MS analysis provides additional information for the structural identification of the metabolites.

2. RESEARCH AIMS

The aims of my PhD were to assess the diversity of bacterial communities and map the changes in the composition of the oral and intestinal microbiota of adult subjects with CFS/ME compared to healthy subjects. My main aim was to point out whether any variation in the bacterial community may be somehow involved in the pathogenesis of CFS/ME and be used in the future for the identification of diagnostic biomarkers.

To investigate the oral and intestinal bacterioma in ME/CFS subjects, I applied a NGS approach. 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 bacterial gene. Amplicons were sequenced using NGS high-throughput platform (MiSeq-Illumina); finally, bioinformatic and statistical analyses, using dedicated software (Mothur, R), were applied for comparing, analyzing and interpreting sequencing data.

In addition, 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 healthy subjects. 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).

3. MATERIALS AND METHODS

3.1 Participant recruitment and sample collection

In this study 105 volunteers were enrolled: 35 CFS patients, 35 patients' relatives without CFS living with patients, and 35 healthy controls.

The recruitment was performed according to the following inclusion criteria: CFS diagnosis according to Fukuda's criteria (Fukuda *et al.*, 1994), age between 18 to 80 years and signature of informed consent.

All participants who had used antibiotics, cortisone and non-steroidal anti-inflammatory drugs, inhibitors of proton pump inhibitors and probiotic drugs in the two months before the recruitment were excluded. Healthy control subjects and patients' relatives who had a previous history of diseases associated with chronic fatigue, bacterial and viral infections, cancer, chronic coronary diseases and allergies were also excluded.

Only the relatives who lived with the patients and shared similar dietary habits with them were enrolled. Age, sex and body mass index were matched in healthy controls.

Twentyfive patients reported gastrointestinal symptoms and showed post-exertional malaise. Moreover, eleven patients also had Irritable Bowel Syndrome (IBS) diagnoses. Neither controls nor patients' relatives reported this syndrome.

In order to measure the extent and severity of fatigue and to evaluate the health status of the patients, CFS subjects completed the Chalder Fatigue scale (*Chalder et al, 1992*) and the MOS SF-36 Health Survey (*Ware et al, 1992*).

The recruitment and sample collection were carried out at the clinic of Laboratory Magi EUREGIO s.c.s of Bozen, thanks to the collaboration of the Italian Association of CFS patients - AMCFS Onlus. Fecal and salivary samples were collected in sterile tubes by the participants themselves and delivered to appointed personnel for storage at -80°C within one hour.

The study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Bozen province. Written informed consent was obtained from all participants.

3.2 Intestinal and oral bacterioma analysis

3.2.1 DNA extraction

Fecal samples. DNA from fecal samples of all participants was extracted using QIAmp DNA Stool Mini kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. First, 180-200 mg of feces were placed in a 2 ml tube to which 1,6 ml ASL buffer was added. The sample was then mixed until it was homogenized and subsequently centrifuged at maximum speed for 1 minute. While the pellet was discarded, 1,4 ml of supernatant were taken and placed in a new 2 ml tube. An InhibitEX tablet was added to the supernatant in order to neutralize PCR inhibitors. The sample was then mixed, incubated at room temperature for 1 minute and centrifuged at maximum speed for 3 minutes. Subsequently, all the supernatant was transferred to a new 1.5 ml tube and centrifuged at maximum speed for 3 minutes. In a new 2 ml tube, 25 μl of proteinase K, 600 μl of supernatant and 600 μl of AL buffer were added. The tube was then vortexed for 15 seconds and incubated at 70°C for 18 minutes. After the incubation, 600 μl of ethanol (96-100%) were added to the lysate. Then, 600 μl of lysate were transferred to a new QIAamp spin column with collection tube and centrifuged at maximum speed for 1 minute. Finally, the tube containing the filtrate was discarded and the QIAamp spin column repositioned in a new collection tube. Subsequently, 500 μl of buffer AW1 were added to the QIAamp spin column which was centrifuged for 1 minute at maximum speed. Then, 500 μl of Buffer AW2 were added to the QIAamp spin column, centrifuged at maximum speed for 3 minutes. The spin column was subsequently inserted into a new 1.5 ml tube. The last phase of the protocol was repeated twice adding each time 100 μl of buffer AE to the QIAamp spin column and incubating at room temperature for 5 minutes. As soon as the incubation ended, the tube containing the spin column was centrifuged at 8000xg for 1 minute. Finally, the QIAamp spin column was discarded while the 1.5 ml tube containing the DNA was stored at -20°C. DNA integrity was tested by agarose gel electrophoresis. DNA integrity was evaluated by agarose gel electrophoresis and DNA concentration measured by Qubit HS dsDNA fluorescence assay (Life Technologies, Carlsbad, CA, USA).

Salivary samples. DNA from salivary samples was extracted by QIAmp DNA Blood Mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions as follows: 1 ml of saliva was collected in a 50 ml tube and added with 4 ml of PBS (Phosphate-Buffered Saline).

After centrifugation at 1800 g for 5 min, the supernatant was discarded and the pellet was resuspended in 180 µl of PBS. After adding 20 µl of QIAGEN Protease and 200 µl of Buffer AL, the sample was incubated at 56°C for 10 min. Then, 200 µl of ethanol (96–100%) were added to the sample, which was loaded on a QIAamp Spin Column in a 2 ml collection tube and centrifuged at 6000 xg for 1 min. The tube containing the filtrate was discarded and the QIAamp spin column was repositioned in a new collection tube. Subsequently, 500 µl of buffer AW1 were added to the QIAamp spin column which was centrifuged for 1 minute at 6000 x g for 1 min. After adding 500 µl of Buffer AW2, the QIAamp spin column was centrifuged again at maximum speed for 3 minutes. The spin column was subsequently inserted into a new 1.5 ml tube. To eluate the DNA, 100 µl of buffer AE were added to the QIAamp spin column, with subsequent incubation at room temperature for 5 minutes. After incubation, the tube containing the spin column was centrifuged at 6000xg for 1 minute. Then, 100 µl of filtered eluate were used for a second elution step. Finally, the QIAamp spin column was discarded, while the 1.5 ml tube containing the DNA was stored at -20°C. DNA integrity was tested by agarose gel electrophoresis. DNA integrity was evaluated by agarose gel electrophoresis and DNA concentration measured by Qubit HS dsDNA fluorescence assay (Life Technologies, Carlsbad, CA, USA).

3.2.2 DNA quantification

DNA concentration was measured by fluorimetric analysis using Qubit® HS dsDNA fluorescence assay (Life Technologies, Carlsbad, CA, USA). The Qubit dsDNA HS (High Sensitivity) protocol for DNA quantification included: the preparation of a Working Solution (W.S.), by diluting the reagent 1: 200 in the Qubit dsDNA HS buffer, and the preparation of the reference standards for instrument calibration, transferring 190 µl of W.S into two test tubes to which then 10 µl of "standard 1" and "standard 2" were added. Two µl of the solution containing extracted DNA were transferred into a 0.5 ml tube adding 198 µl of W.S to obtain a final volume of 200 µl. The samples were exposed to a light source to measure the DNA concentration. The average of three measures was calculated. To calculate the real concentration of the sample the following formula was applied: Sample value = QF value * (200/x), where QF is the mean value displayed on the fluorimeter and x is microliters of extracted DNA (1-20 µl) that were measured. Once the real quantity of extracted DNA was calculated, the DNA of all samples were diluted and brought to the final concentration of 1 ng/µl for the next step.

3.2.3 16S rRNA gene amplification and Illumina sequencing

The PCR amplification of both the V3 and V4 regions of 16S rRNA was conducted using the indexed primers 343 F (5′-TACGGRAGGCAGCAG-3′) and 802 R (5′-TACNVGGGTWTCTAATCC-3′), as described in *Spigni et al*. (2018).

PCR reactions were performed in 25 μL containing 12,5 μL of Phusion Flash High-Fidelity Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 0,5 μM of each primer, 1 ng of DNA template and PCR nuclease-free water. To analyze several amplicon samples simultaneously in the same sequencing run, a multiplexing strategy was performed. An extension of nine nucleic acids was added to the 5' end of the forward primer, with the first seven bases acting as a tag, to identify each sample unequivocally, and the other two bases acting as a linker.

The two step-PCR method described in *Berry et al.* (2011) was adopted, with a first PCR step of 25 cycles using untagged primers, and a second PCR step of 8 cycles with tagged primers and 1 μL of first step products used as template. The PCR termal profile was the same in the two steps: 30 seconds of denaturation at 94°C, 30 seconds of primer annealing at 50°C and 30 seconds of primer elongation at 72 °C, followed by a final elongation step of 10 minutes at 72°C.

The final PCR products, approximately 450 bp long, were verified by electrophoresis on 1% agarose gel and the concentrations of amplicons were measured by Qubit® HS dsDNA fluorescence assay (Life Technologies, Carlsbad, CA, USA).

Equimolar concentrations of amplicons (35 ng/μL for faecal samples and 30 ng/μL for salivary samples) were pooled and the final pool was then purified with the SPRI (Solid Phase Reverse Immobilization Method) using Agencourt AMPure XP kit (Beckman Coulter, Italy, Milano). The pool was finally sent to Fasteris Company (Geneva, Switzerland) for the amplicon library preparation and for paired-end sequencing (2 bp x 300 bp) on MiSeq Illumina platform (Illumina Inc, San Diego, CA), operating with V3 chemistry.

3.2.4 Electrophoresis on agarose gel

The correct amplification of bacterial DNA was evaluated by electrophoretic running of all samples on 1% agarose gel. This technique allows the separation of charged molecules, thanks to the application of an electric field. The 1% agarose gel (ThermoFisher, Waltham, MA USA) was prepared dissolving 1 g of agarose in 100 ml TAE buffer (Biorad, Hercules, CA USA) and finally adding 5μl of Sybr Safe DNA gel stain (Invitrogen, Carlsbad, CA USA). Two μl of

KAPA Loading Dye (6X) were added to 10 μl of amplified product. To verify the presence of the expected products (459 bp), bands were visualized using a UV transilluminator.

3.2.5 Dataset preparation and data analysis

Illumina sequencing data were pre-processed for OTU- and taxonomy-based analyses. Raw paired reads were assembled with the "pandaseq" script (Masella *et al.*, 2012), allowing at least 30 bp of overlap between the read pairs and a maximum of two mismatches. Sequences were demultiplexed and quality-controlled with the fastx-toolkit, according to sample indexes (http://hannonlab.cshl.edu/fastx_toolkit/).

Mothur v.1.39.5 software (Schloss *et al.*, 2009) was used in order to remove sequences with large homopolymers (≥10), sequences that did not align within the targeted V3-V4 region, chimeric sequences (Edgar *et al.*, 2011), and sequences that were not classified as bacterial after alignment against the Mothur version of the RDP training data set. The resulting high-quality sequences were analyzed with Mothur and R (*http://www.r-project.org/*) following both the OTU- and taxonomy-based approach. By the OTU approach, sequences were first aligned against the SILVA reference database for bacteria (Pruesse *et al.*, 2007) using the NAST algorithm and a kmer approach (De Santis *et al.*, 2006; Schloss *et al.*, 2010), and then clustered in OTUs (Operational Taxonomic Units) with 97% similarity using the average linkage algorithm. OTUs were classified at taxonomical levels by alignment against the Greengenes database (McDonald *et al.*, 2012).

Based on OTU matrixes, the α and β diversity indices were calculated using Mothur and R software. α-diversity is used to measure the diversity within a sample, calculating a index for each sample. Different metrics based on richness, abundance and "evenness" of species were applied to calculate diversity indices: Observed species, Chao1 and inverse Simpson. Observed species index was calculated counting only unique OTUs (richness) in each sample. Chao1 and Simpson's indices were calculated based on richness and distribution of species (evenness) in each sample. Evenness (or equitability) refers to how equally abundant species in a sample are. β-diversity is used to estimate the diversity between samples and provides a measure of the distance between each sample pair. β-diversity is calculated for every pair of samples to generate a dissimilarity matrix. Bray-Curtis metric, a non-phylogeny based method that takes abundance of species into account, was applied to calculate the indices of dissimilarity.

The good coverage estimate was calculated to evaluate the percentage of diversity captured by sequencing. The most abundant OTUs identified were confirmed by BLAST (Basic Local Alignment Search) searches against the RDP database.

Statistical analyses were carried out on OTU matrixes by using Mothur and R softwares and included hierarchical clustering analyses with the average linkage algorithm at different taxonomic levels, principal component analysis (PCA) and canonical correspondence analysis (CCA). Metastats, a statistical software based on non-parametric t-test, was used to compare OTUs between groups (Paulson *et al.*, 2011). ANOVA, t test and post hoc Bonferroni correction were applied to test for any significant difference in the relative abundances of oral and intestinal bacterial taxa between groups.

By PCA data from the β-diversity distance matrix were represented in two dimensional plots (PC1 and PC2). The distances between the samples are used to define similarities and differences between samples.

Multivariate CCA was performed on bacterial OTUs abundance table in order to assess if health state was significantly affecting the relative distribution of OTUs across individuals.

3.3 Analysis of fecal metabolites by UPLC - MS

3.3.1 Instrument and sample preparation

The metabolic analysis was performed on selected fecal samples by a UPLC (Ultra Performance Liquid Chromatography) chromatographic system, characterized by short analytical time and high chromatographic resolution, interfaced with a high-resolution Q-ToF mass spectrometer (MS).

After collection, the fecal samples were aliquoted into 2 ml tubes and then frozen at -80 °C. One gram of each fecal sample was put into a 15 ml tube and then 10 ml of methanol (80%) and 0,1 ml of formic acid were added for the extracting the compounds. After an overnight incubation, 15 mL tubes were centrifuged at 20000 xg for 10 minutes in order to precipitate insoluble materials. Subsequently, 1 mL of supernatant was filtered using a 0,22 μm cellulose filter and transferred into glass vials for chromatografic analysis. Quality controls (QC) were prepared by mixing an aliquot (40 μL) of each sample.

3.3.2 Chromatographic and spectroscopic analysis

Samples were analyzed using a chromatographic column at inverse phase. In the polar mobile phase, eluents used for the analysis were: solvent A: H_2O + 1% Formic Acid; and solvent B: Acetonitrile + 1% Formic Acid. The analysis was carried out in gradient by increasing progressively the volume of one solvent while decreasing the volume of the second solvent. The non polar stationary phase was constituted by a long chain hydrocarbon anchored to a silice support.

MS analysis was performed by a high-sensitivity and high-resolution Q-ToF mass spectrometer, capable to separate the metabolites based on their exact mass in order to identify the different compounds. An electrospray ionizator (ESI) was used for the ionization of compounds and the analyses were performed in positive ionization.

All the samples were analysed in triplicate and injected in three random sequences in order to avoid any classification bias due to analytical conditions. QC were analysed together with the samples to verify the analytical reproducibility. Furthermore, to highlight possible contaminations, controls consisting only of solvent were also inserted.

3.3.3 UPLC-MS data extraction and statistical analyses

Data extraction was performed through chromatogram analysis using Mass Profinder B.06 and Mass Profiler Professional software (Agilent, Santa Clara, CA USA). Optimization parameters for data extraction were obtained through the preliminary QC analysis. After extraction, data were analyzed according to an untargeted approach and processed in order to obtain a data set of identified variables. The variables were normalized on the baseline (median) and by means of a normalization algorithm (percentile shift at 75%). The data set was filtered in order to eliminate potentially uninteresting variables for subsequent analyses.

The resulting variables were treated with "Pareto" scaling and centering on the average. A multivariate PCA model was used to describe the data set. The PCA is an unsupervised method which allows to obtain latent variables (Principal Components) which describe the variability among samples. The PCA permits to simplify the information contained in the original data matrix, and explore the distribution and similarity of the samples' metabolic profiles highlighting the presence of clusters. PLS–DA (Partial Least Square - Discriminant Analysis) and OPLS-DA (Orthogonal Latent Structure - Discriminant Analysis) classification models were then applied to the data set. These supervised methods, based on previous knowledge of sample classification, allow to identify more clearly which experimental information is the most useful for discriminating between two or more groups. In the OPLS-DA model this information is summarized in a small number of predictive scores resulting from a combination of the measured variables. A VIP (Variable Importance in Projection) score is a measure of a variable's importance in the OPLS-DA model and summarizes each variable's contribution to the model. The VIP score is calculated as the weighted sum of the squared correlations between OPLS-DA components and the original variable. Weights represent the percentage variation explained by the PLS-DA component in the model. Compounds with a VIP score greater than 1 are those which contribute the most to group discrimination in the OPLS-DA model.

Relative concentrations (fold changes) of metabolites were determined by calculating the ratio of the average MS ion intensities (peak areas) in the experimental groups. To determine statistical significance of fold changes, Student's t-test (two-tailed) and post hoc Bonferroni correction were applied to peak area values of biological replicates to assess any significant difference between groups. A p-value threshold of 0.05 was used.

The model obtained can be interpreted in terms of the set of variables which characterize a specific group. The permutation test was applied to confirm the validity of the model and exclude random associations.

4. RESULTS

4.1 Characteristics of the study population

The study was carried out on a total of 105 subjects: 35 patients (P) and 70 control subjects. The healthy control population was constituted of two distinct groups: 35 patients' relatives (R) without CFS/ME and 35 healthy subjects (C) not belonging to patients' family (Table 3).

Table 3. Characteristics of study population.

		CFS/ME	RELATIVES	CONTROLS
Gender	Female	26	16	26
	Male	9	19	9
Ethnicity/Nationality		Caucasian/Italian	Caucasian/Italian	Caucasian/Italian
Geographic area		Northern Italy	Northern Italy	Northern Italy
Age	Mean ± SD	46,4 ± 16,1	54,4 ± 15,9	55,2 ± 18
	Median	49	55	58
BMI	Mean ± SD	23,1 ± 4.4	25,5 ± 5	23,5 ± 4,7
	Median	22.6	25.3	22
Smoke	Smoker	35 (100%)	35 (100%)	35 (100%)
	No smoker	-	-	-
Diet	Omnivorous	35 (100%)	35 (100%)	35 (100%)
	Vegetarian	-	-	-
	Vegan	-	-	-
GI symptoms	Yes	25 (71%)	7 (20%)	-
	No	10 (29%)	28 (80%)	35 (100%)
Post-exertional malaise	Yes	30 (85%)	2 (5%)	-
	No	5 (15%)	33 (95%)	35 (100%)
IBS co-morbidity	Yes	11 (31%)	-	-
	No	24 (69%)	35 (100%)	35 (100%)
Chalder Fatigue Scale	Range: 0-33	24,9 ± 5.1	-	-
SF-36 Health Survey	Range (%): 0-100		-	-
Physical functioning:		52% ± 2,4	-	-
Role limitations due to physical health:		13% ± 3,1	-	-
Role limitations due to emotional problems:		69% ± 4,7	-	-
Energy/fatigue:		23% ± 1,5	-	-
Emotional well-being:		65% ± 1,2	-	-
Social functioning:		33% ± 2,2	-	-
Pain:		49% ± 2,8	-	-
General health:		24% ± 1,2	-	-
Health change:		50% ± 2,5	-	-

Twenty-six CFS/ME patients were females and nine were males, with a mean age of 46,4 ± 16,1 years and a BMI of 23,1 ± 4,4. Twentyfive patients (71%) self-reported gastrointestinal disturbances and 11 of them (31%) showed IBS comorbidity. The majority of the patients, 30 (85%), indicated a post-exertional malaise.

Patients' relatives had a mean age of 54,4 ± 15,9 years and a BMI of 25,5 ± 5. Sixteen relatives were females and 19 were males. The control group included 26 females and 9 males, with a mean age of 55,2 ± 18 years and a BMI of 23,5 ± 4,7.

Seven patients' relatives (20%) reported gastrointestinal symptoms, while only two relatives (5%) showed post-exertional malaise. No external controls and patients' relatives reported IBS diagnosis. All participants were Caucasian race, followed an omnivorous diet and belonged to the same geographical area (Northern Italy). No one was a smoker.

CFS subjects completed the Chalder Fatigue scale (Chalder *et al.*, 1993) and the MOS SF-36 Health Survey (Ware *et al.*, 1992).

4.2 Sequencing data analysis of intestinal and oral bacterial communities

Sequences with large homopolymers (≥10), sequences <380 bp, chimeric sequences, sequences that did not align within the targeted V3-V4 region, and sequences that were not classified as bacterial after alignment against RDP database were removed.

After the assembly and demultiplexing of the Illumina paired-end sequences, a total of 9,967,562 and 4,774,706 raw sequences was obtained for fecal and salivary samples respectively. After quality trimming and chimera checking, these were reduced to 8,788,426 and 3,508,491 filtered sequences. To avoid biases in diversity estimates due to differences in the number of sequences per sample, rarefaction to a common minimum number of 6,100 and 5,200 sequences per sample, which represent the number of sequences of the lowest populated sample, was performed for fecal and salivary samples respectively.

After the rarefaction step, 481,803 high quality sequences for fecal samples and 585,579 high-quality sequences for salivary samples remained for further analyses.

A high average Good's coverage (98,53% ± 0,64%) was obtained for fecal samples, highlighting that most of bacterial diversity in the samples was represented. Conversely, in salivary samples the Good's coverage index was lower (79,90% ± 1,94%).

Subsequently, the reads were clustered in OTUs (Operational Taxonomic Units) at 97% similarity, without any sub-filtering for rare OTUs, and resulted in a total of 4231 OTUs for fecal samples and 78000 OTUs for salivary samples, with an average number of 207 and 1235 OTUs per sample respectively (Tab. 4).

Both in feces and saliva, a lower OTUs number was observed in patients' relatives comparing to two other groups, while no substantial difference was between CFS/ME patients and the external control group.

Table 4. OTUs average number of intestinal and oral microbiota in CFS patients, relatives and controls. *Comparison of OTUs numbers between the three groups. Univariate (ANOVA) test is applied in order to assess significant differences between groups (P < 0.05).*

	OTUs average number			**p-value (ANOVA)**
	CFS PATIENTS	**RELATIVES**	**CONTROLS**	
Feces	216 ± 78,04	189 ± 74,06	221 ± 60,84	0,27
Saliva	1258 ± 97,14	1217 ± 91,50	1236 ± 139,43	0,37

Based on the number of OTUs identified, to estimate the efficiency of the sequencing analysis the percentages of bacterial sequences correctly classified was calculated at different taxonomic levels for fecal and salivary samples (Fig. 9 A-B).

In fecal samples all sequences were correctly classified up to the order-family levels (99%), with a progressive reduction at genus (77,6%) and species (31,7%) levels (Fig. 9 A). For salivary samples (Fig. 9 B), all sequences were correctly classified up to the class level (99,3%), while percentages were slightly reduced going down to the order (96,3%), family (94,8%), genus (93,4%) and species level (60,6%).

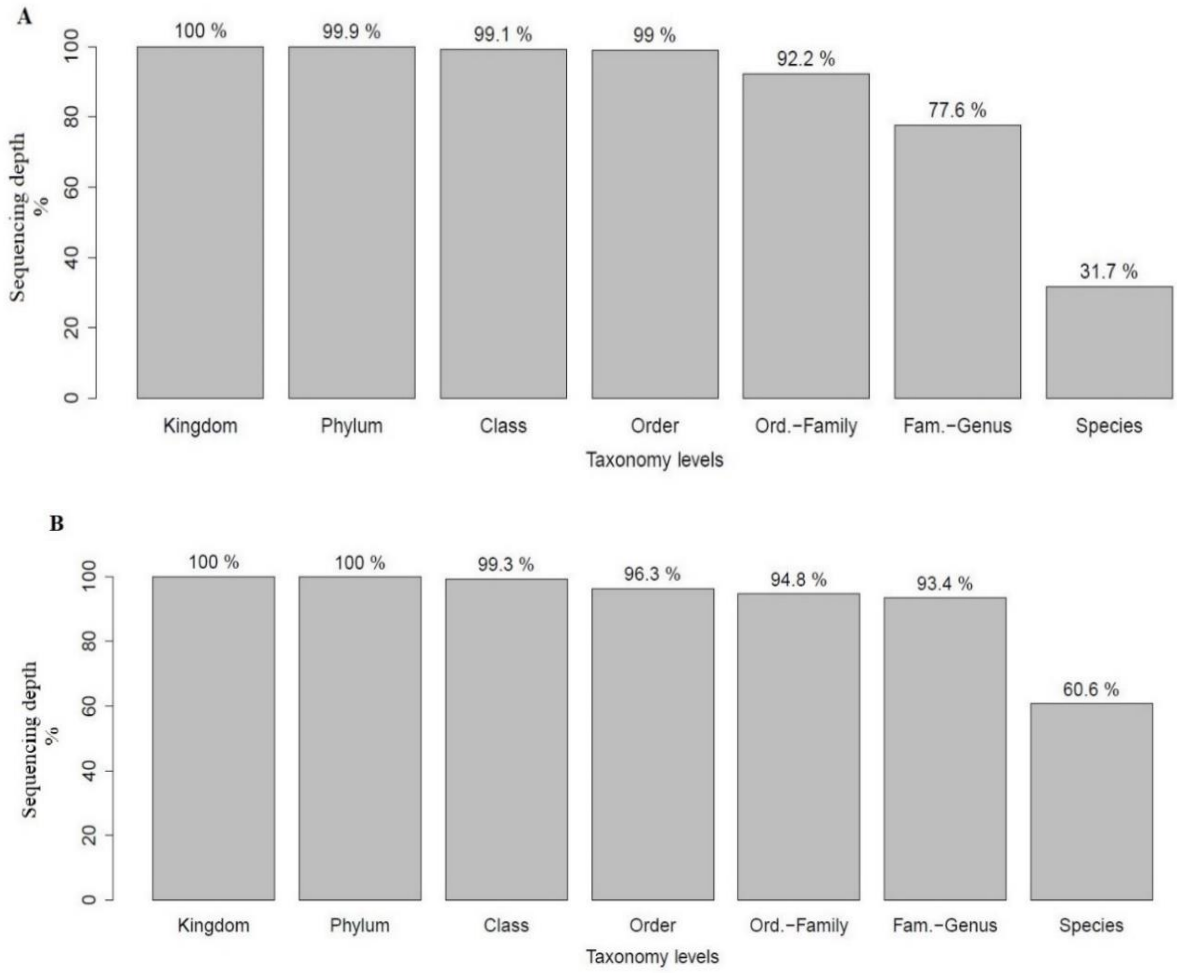

Figure 9 A-B. Percentages of bacterial sequences correctly classified at different taxonomic depth. *Data refer to sequences identified as bacterial after PCR amplification of the V3-V4 16S rRNA region in fecal (A) and salivary (B) samples at different taxonomical levels.*

4.2.1 Intestinal microbiota diversity

The OTUs table was used for the subsequent α- and β-diversity analyses. The diversity within the intestinal bacterial community in all participants was evaluated determining different α-diversity indices (Table 5). Bacterial richness and evenness were assessed basing on Observed species (total OTUs), on Chao1 and Simpson's evenness diversity indices.

Table 5. OTU-based diversity indexes in fecal samples of the experimental groups of subjects. *The table shows the total number of OTUs, (b) Chao index and (c) Simpson's evenness indexes in CFS patients, patients' relatives and external control group respectively. Univariate (ANOVA) test is applied in order to assess significant differences between groups (P < 0.05).*

α-diversity indices	**P** (mean)	**R** (mean)	**C** (mean)	**p- value (ANOVA)**
Chao1	453,44 ± 194,73	366,96 ± 155,90	422,03 ± 151,23	0,14
Observed S	215,60 ± 78,03	189,26 ± 74,06	221,43 ± 60,83	0,25
Simpson	17,77 ± 11,12	15,62 ± 9,08	13,35 ± 7,34	0,31

No significant difference in terms of total measured OTUs was recorded among groups despite the patients' relatives showed a slightly lower richness. Chao1 and Simpson's evenness indices resulted slightly increased in CFS/ME patients, although the differences were not statistically significant.

Multivariate β-diversity analyses were carried out by Principal Component Analysis (PCA, Fig. 10) and by Canonical Correspondance Analysis (CCA, Fig. 11) in order to evaluate the dissimilarity between the intestinal microbiome of all participants.

No difference in β-diversity was recorded among groups. According to β-diversity indices, PCA results showed a full overlapping of CFS/ME patients, their relatives and external controls.

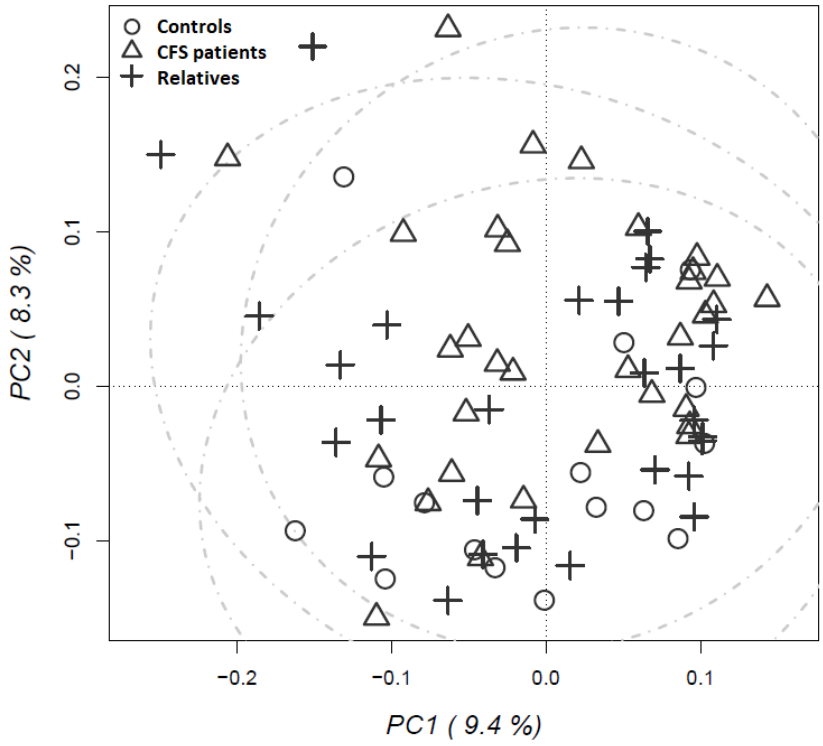

Figure 10. Principal component analysis (PCA) of fecal microbiota compositions in the experimental groups of subjects. *PCA was performed based on abundance of classified OTUs in CFS patients, patients' relatives and external healthy controls, respectively. The percentages on each axis indicate the variation in the samples. Individuals are labeled according to the three groups studied.*

Microbial diversity was further examined by CCA analysis in order to assess if healthy state might influence the relative distribution of OTUs across individuals.

Three partially overlapping clusters were recorded (p = 0,003), although the model explained only 4% of variance. CFS/ME individuals were separated from their relatives and the external control group along the first major axis, while the second axis separated CFS/ME patients and external controls from patients' relatives.

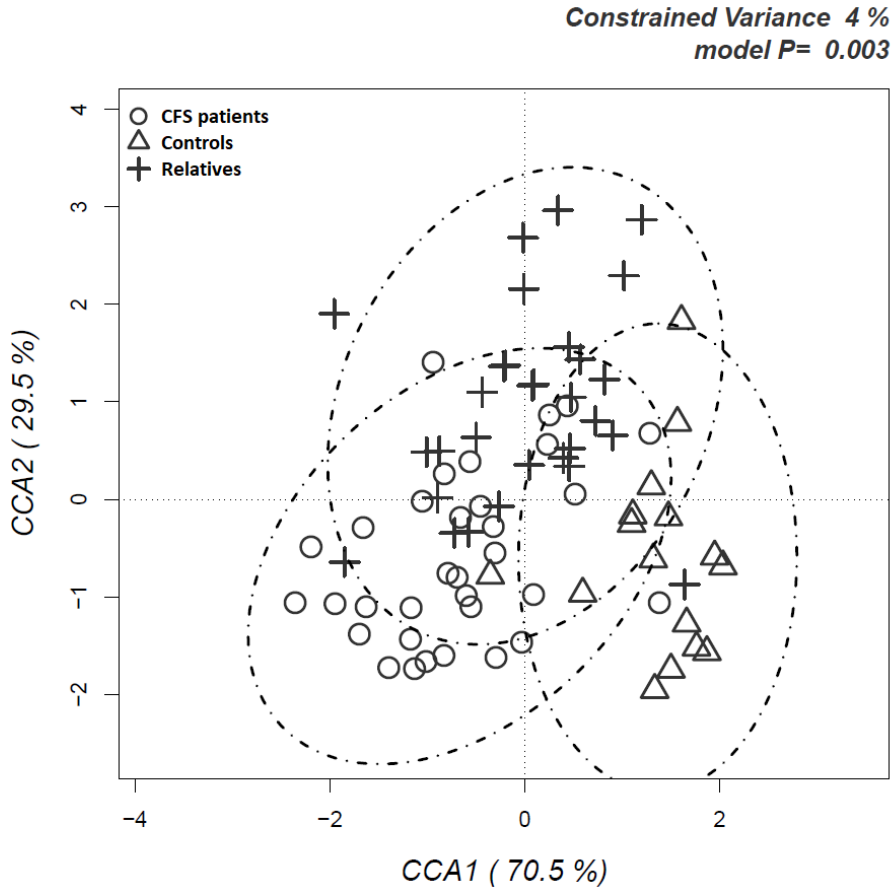

Figure 11. Canonical correspondence analysis (CCA) of fecal microbiota compositions in the experimental groups of subjects. *CCA was performed based on abundance of classified OTUs in CFS patients, patients' relatives and external healthy controls, respectively. The plot shows that the disease status is a significant source of variability in bacterial communities, explaining 4% of the variance in fecal microbiota. The percentages on each axis indicate the variation in the samples. Individuals are labeled according to the three groups studied.*

For each sequence, hierarchical clustering analysis was performed at different taxonomic levels in order to determine the relative distribution of different bacterial taxa among samples and assess the grouping of individuals. At the phylum level (Fig. 12 A) *Firmicutes* predominated, followed by *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*. Some CFS/ME patients clustered separately because of a reduction in the abundance of *Firmicutes* mirrored by an increase in the relative abundance of *Bacteroidetes*. However, a clear separation of all CFS/ME patients from the other two groups was not observed.

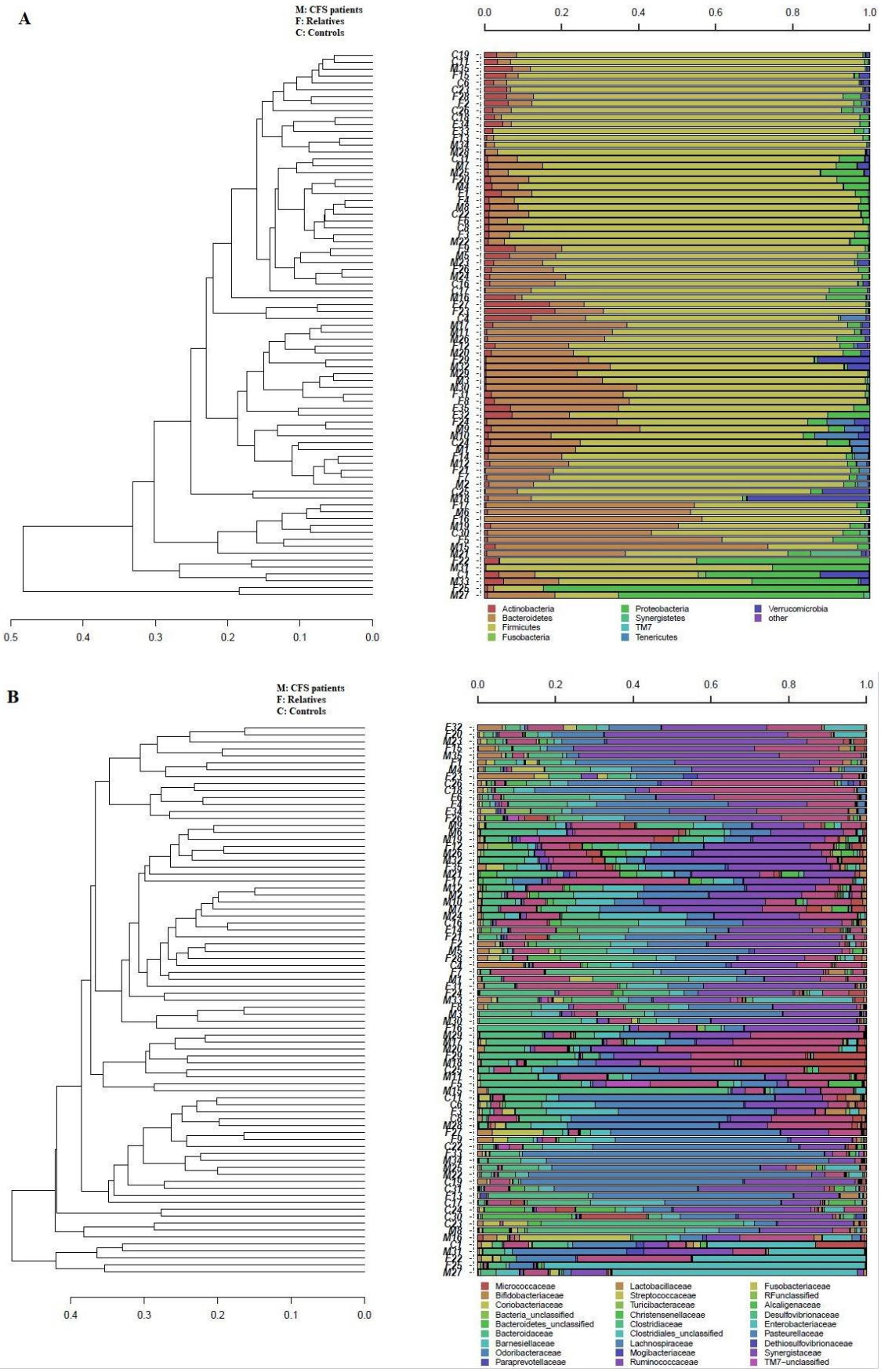

Figure 12. Hierarchical clustering of classified sequences at phylum (A) and family (B) classification levels. *The three experimental groups are indicated as M (CFS patients), F (patients' relatives), and C (external healthy controls). Bars of different colors indicate the relative percentage of bacterial phyla identified in each fecal sample. Only taxa participating with ≥5% in at least one sample are shown, while taxa with lower participations were added to the "other" sequence group. Similar samples were clustered using the average linkage algorithm.*

When OTUs were classified at the family level, a partial grouping of some CFS/ME patients (M12-M2-M10-M7-M24) was observed, showing a higher relative proportion of *Bacteroidaceae* and a lower abundance of *Lachnospiraceae* (Fig 12 B).

Metastats analysis was performed in order to determine OTUs whose relative abundance was significantly different between groups (Fig.13). Considering the 48 most aboundant OTUs, which represented the 95% of the total biodiversity in the analyzed samples, most were represented by members belonging to the *Firmicutes* phylum. Their relative abundance was lower in CFS/ME patients and in their relatives compared to external control subjects.

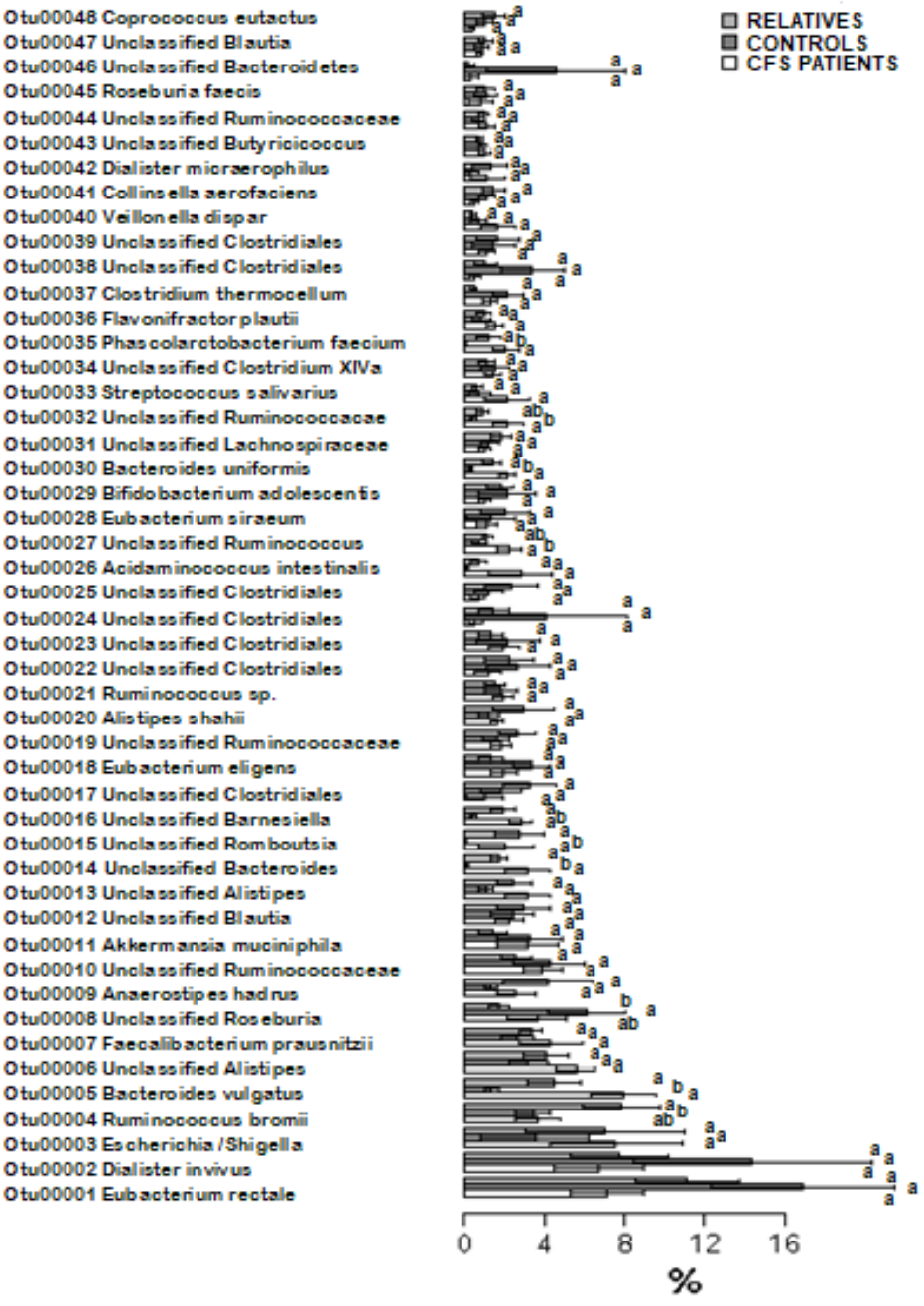

Figure 13. Differential relative abundance of specific OTUs across the three experimental groups. *Metastats model was applied on the 48 most abundant OTUs. Significant differences are highlighted by different minor letters (P < 0.05).*

OTUs classified respectively as *Bacteroides vulgatus* (OTU00005), *unclassified Bacteroides* (OTU00014), *unclassified Romboutsia* (OTU00015), *unclassified Barnesiella* (OTU00016), *unclassified Ruminococcus* (OTU00027), *Bacteroides uniformis* (OTU00030), *unclassified Ruminococcaceae* (OTU00032), *Phascolarctobacterium faecium* (OTU00035) were significantly more abundant in CFS/ME patients and their relatives, while *unclassified Roseburia* (OTU00008) was significantly less abundant in patients' relatives than in controls. In addition, *Ruminococcus bromii* (OTU00004) was significantly more abundant in the patients' relatives.

Classification of OTUs at different taxonomic levels was carried out in order to evaluate the relative abundance of different bacterial taxa between samples. The significant differences between experimental groups in the relative abundances of intestinal bacterial taxa are reported in Table 6.

At phylum level, CFS/ME patients, their relatives and external control group showed that their intestinal bacteria clustered within five main bacterial phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia* (Fig. 14).

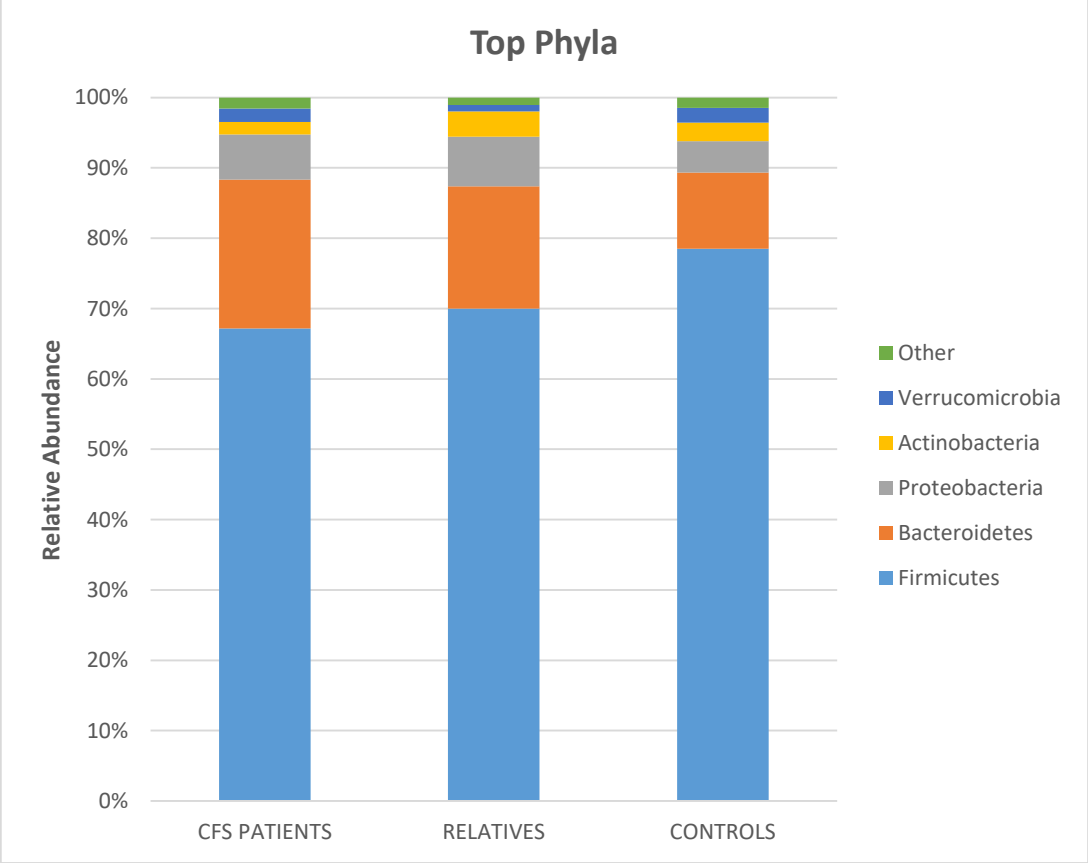

Figure 14. Compositions of the intestinal microbiota from CFS patients, their relatives and controls at phylum level. *Only taxa ≥1% in at least one sample are shown, while taxa with lower participations were added to the "other" sequence group.*

A lower abundance of *Firmicutes* (P = 67,17% vs R = 69,99% vs C = 78,53%) and a significant increase in *Bacteroidetes* (P = 21,16% vs R = 17,40% vs C = 10,80%) were observed in CFS/ME patients (p = 0,0005) compared to external controls. In addition, a decrease in *Actinobacteria* prevalence (P = 1,76% vs R = 3,60% vs C = 2,66%) and a higher proportion of *Proteobacteria* (P= 6,42% e R= 7,04% vs C= 4,45%) were also detected in CFS/ME patients and in their relatives (not statistically significant).

A change in *Firmicutes*/*Bacteroidetes* ratio was observed (Fig. 15), with a reduction of about 60% in CFS/ME patients and of 50% in their relatives compared to the external control group (not statistically significant).

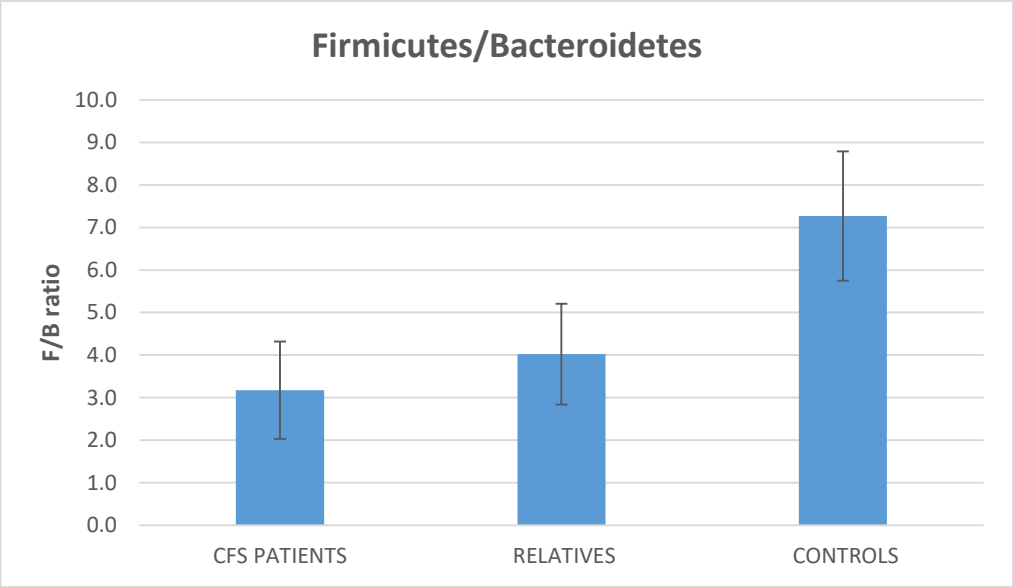

Figure 15. Firmicutes/Bacteroidetes (F/B) ratio in fecal samples across the three experimental groups. *Comparison of F/B ratio between CFS patients, their relatives and external controls.*

In total seven orders with relative aboundance ≥ 1% in at least one sample were found, some of which showed significant differences among groups (Fig. 16). Among the most interesting variations, a lower abundance of *Clostridiales* (P = 64,26% vs R = 67,77% vs C = 76,95%) was observed in CFS/ME patients (p = 0,023) compared to external controls; conversely, *Bacteroidales* (P = 20,70% vs R = 17,12% vs C = 8,63%) were significantly more present in patients (p = 0,0005) and their relatives (p = 0,015).

Other differences, not statistically significant, were observed for the orders *Enterobacteriales* (P = 4,71% vs R = 5,51% C = 2,19%), whose presence in CFS/ME patients and in their relatives was approximately twice compared to that found in the external controls, *Actobacillales* (P = 2,02% vs R = 0,74% vs C = 0,64%), which increased only in CFS/ME patients, and

Bifidobacteriales (P = 0,96 % vs R = 1,82% vs C = 1,39%), which were less abundant in CFS/ME patients compared to their relatives and external controls.

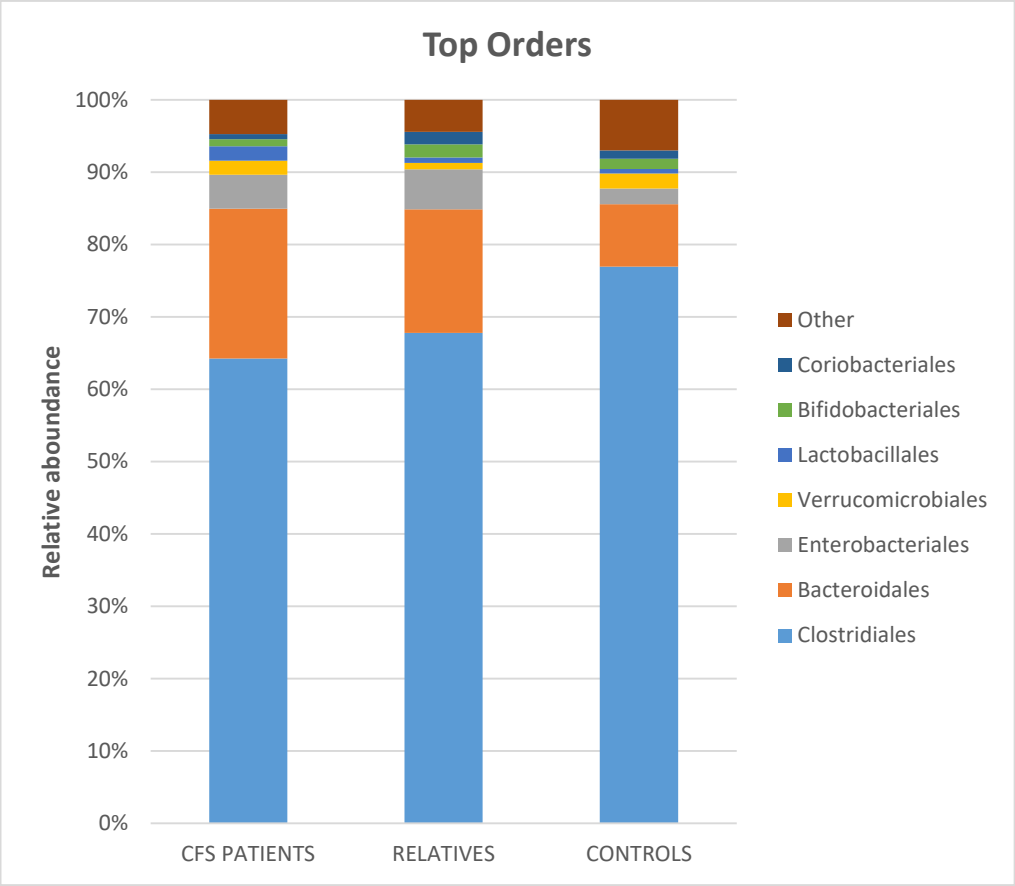

Figure 16. Compositions of the intestinal microbiota from CFS patients, relatives and controls at order level*. Only taxa ≥1% in at least one sample are shown, while taxa with lower participations were added to the "other" sequence group.*

At family level, the ten most aboundant families in the three experimental groups were *Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae*, *Clostridiaceae*, *Bacteroidaceae*, *Rikenellaceae*, *Enterobacteriaceae*, *Streptococcaceae*, *Christensenellaceae*, *Coriobacteriaceae* and *Barnesiellaceae* (Fig. 17). Of these, *Lachnospiraceae* (P = 18,07% vs R = 20,56% vs C = 30,11%) were reduced in CFS/ME patients (p = 0,034) compared to external controls; conversely, *Bacteroidaceae* family (P = 9,93% vs R = 6,59% vs C = 1,27%) were about 10 times more abundant in CFS/ME patients (p = 0,0002) and 6 times in their relatives (p = 0,003) respect to external controls. Another member of *Bacteroidales*, namely *Barnesiellaceae* (P = 1,71% vs R = 1,15% vs C = 0,27%), was significantly increased in both patients (p = 0,0005) and their relatives (p = 0,0089). Not statistically significant differences emerged for the families *Rikenellaceae* (P = 7,03% vs R = 7,01% vs C = 4,89%), *Enterobacteriaceae* (P = 4,71% vs R = 5,51% vs C = 2,19%), belonging to the *Proteobacteria* phylum, *Streptococcaceae* (P = 1,88% vs R = 0,65% vs C = 0,63%), *Christensenellaceae* (P =

0,90% vs R = 0,99% vs C = 1,84%), and *Coriobacteriaceae* (P = 0,69% vs R = 1,72% vs C = 1,18%).

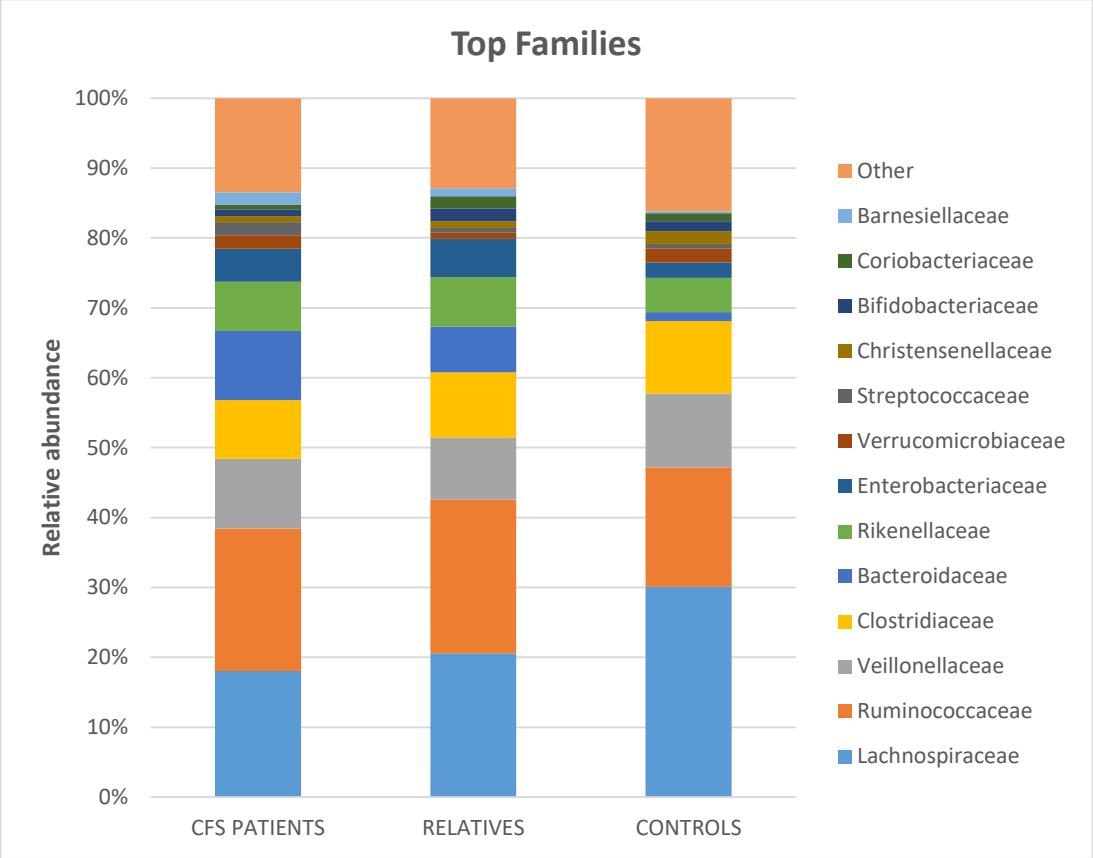

Figure 17. Compositions of the intestinal microbiota from CFS patients, relatives and controls at family level. *Only taxa ≥1% in at least one sample are shown, while taxa with lower participations were added to the "other" sequence group.*

At genus level, *Anaerostipes* (P = 0,18% vs R = 0,47% vs C = 1,15%), was significantly less abundant in both CFS/ME patients (p = 0,02) and their relatives (p = 0,032) compared to external controls (Fig. 18). In contrast, *Bacteroides* (P = 9,93% vs R = 6,59% vs C = 1,27%) and *Phascolarctobacterium* (P = 1,17% vs R = 0,76% vs C = 0,15%) were about 10 times more abundant in CFS/ME patients (p = 0,0002 and 0,004, respectively) and *Bacteroides* was about 6 times more abundant in patients' relatives (p = 0,003).

Also *Ruminococcus* (P = 6,60% vs R = 11,48% vs C = 8,21%) was significantly more abundant in patients' relatives (p = 0,0194). *Roseburia* (M = 6,61% vs F = 6,89% vs C = 12,50%), *Clostridium* (P = 6,85% vs R = 7,03% vs C = 9,85%), *Dialiste*r (M = 5,23% vs F = 6,63% vs C = 9,35%), *Coprococcus* (P = 3,54% vs R = 5,35% C = 6,60%) and *Lachnospira* (P = 1,59% vs R = 1,06% vs C = 3,60%), all belonging to *Clostridiales* order, exhibited the largest, although not statistically significant, differences among groups, with the lowest values in CFS/ME subjects. Conversely, *Faecalibacterium* (P = 5,01% vs R = 3,91% vs C = 2,31%), *Enterobacter*

(P = 4,52% vs R = 5,14% vs C = 2,14%), *Acidaminococcus* (P = 1,99% vs R = 0,66% vs C = 0,11%) and *Streptococcus* (P = 1,88% vs R = 0,64% vs C = 0,63%) were more frequent in CFS/ME patients (not statistically significant). *SMB53* (P = 1.15% vs R = 1.86% vs C = 0.05%), belonging to *Clostridiaceae* family, was substantially absent in external controls, although it was not statistically significant.

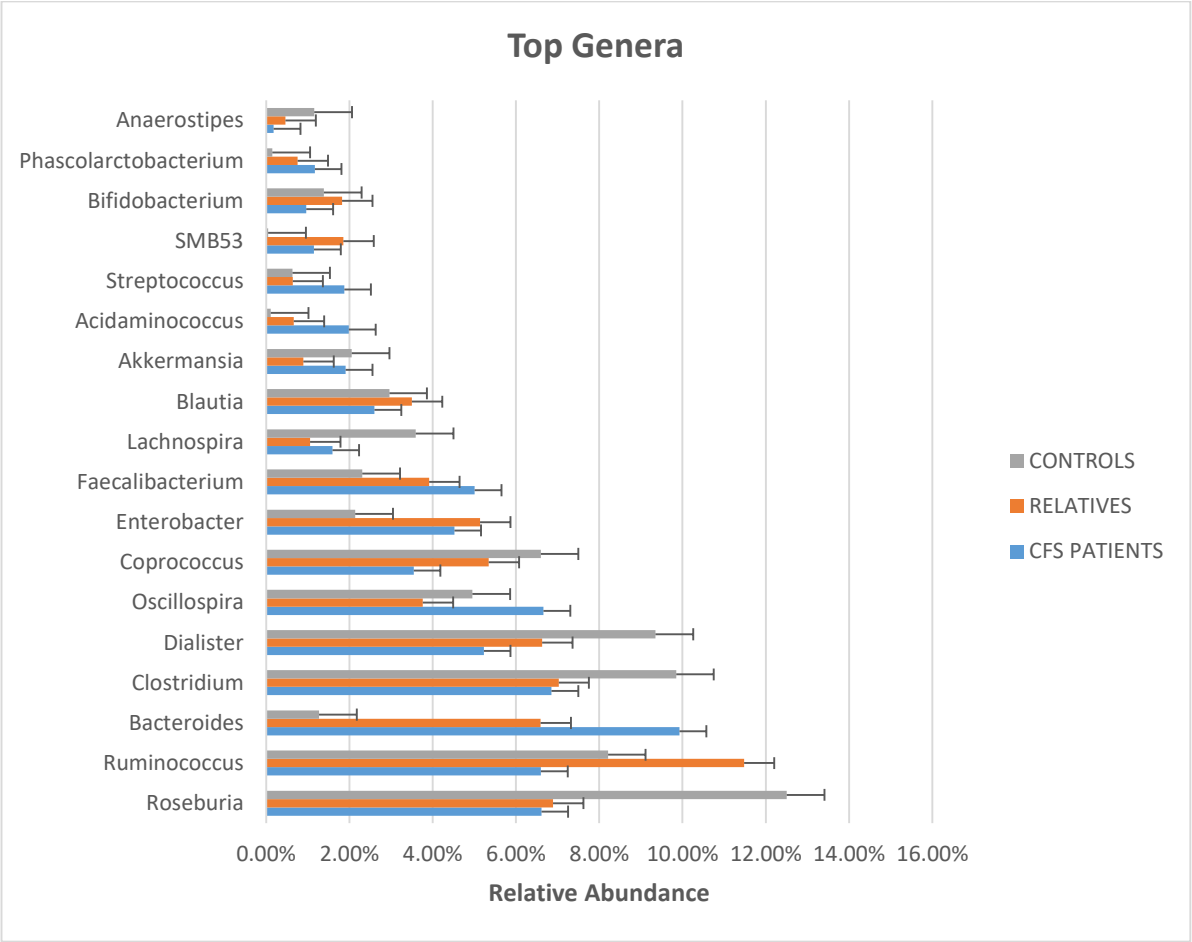

Figure 18. Compositions of the intestinal microbiota from CFS patients, relatives and controls at genus level. *Only taxa ≥1% in at least one sample are shown.*

Only 30% of the sample could be classified to species level (Fig. 19). Significant differences were observed for species *Bacteroides ovatus* (P = 0,98% vs R = 1,01% vs C = 0,10%) and *Bacteroides uniformis* (P = 1,23% vs R = 0,91% vs C = 0,14%), which were about 10 times more abundant in both CFS/ME patients (p = 0,0001 and p = 0,00005, respectively) and their relatives (p = 0,0021 and p = 0,0169, respectively) than in external controls.

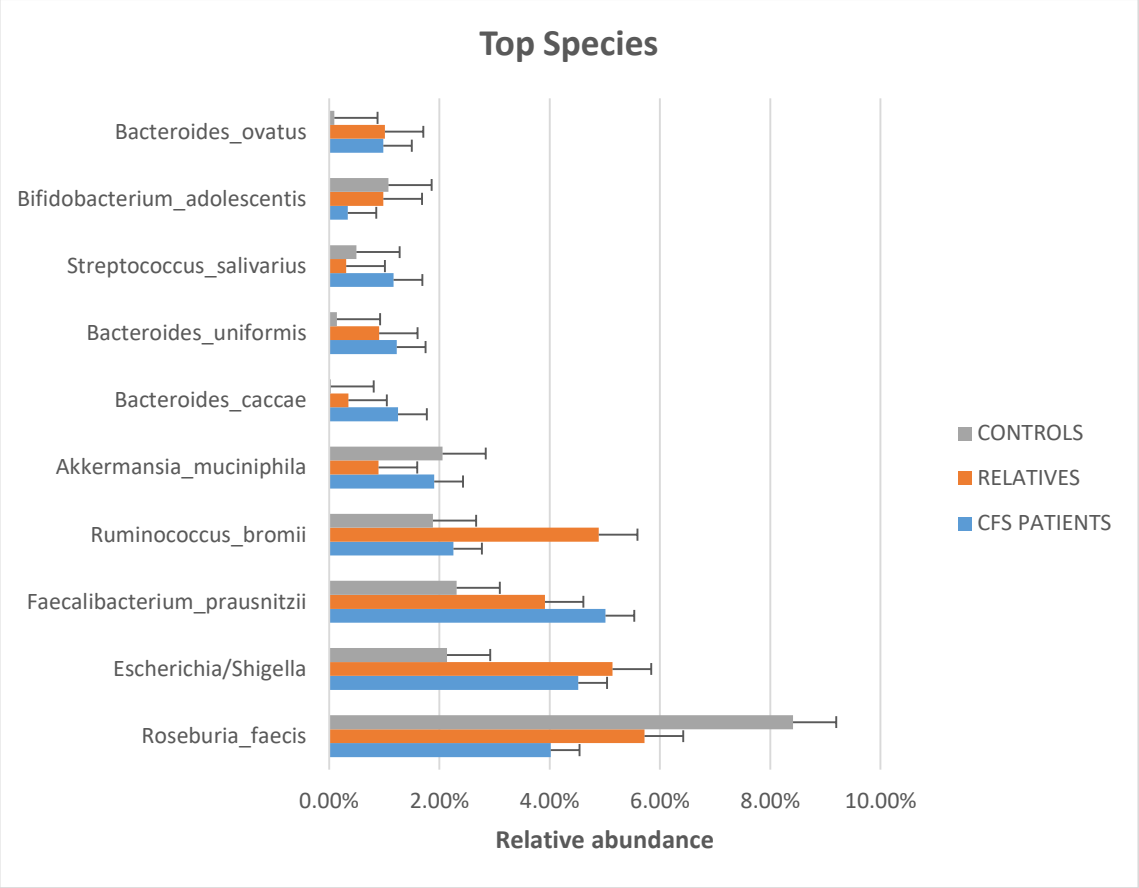

Figure 19. Compositions of the intestinal microbiota from CFS patients, relatives and controls at species level. *Only taxa ≥1% in at least one sample are shown.*

Ruminococcus bromii (P = 2,25% vs R = 4,89% vs C = 1,89%) showed a significant increase only in patients' relatives compared to external controls (p = 0,0314).

Not significant variation in relative abundance were recorded for *Roseburia faecis* (P = 4,02% vs R = 5,72% vs C = 8,41%), *Bifidobacterium adolescentis* (P = 0,34% vs R = 0,98% vs C = 1,08%), *Bacteroides caccae* (P = 1,25% vs R = 0,35% vs C = 0,03%), *Faecalibacterium prausnitzii* (P = 5,01% vs R = 3,91% vs C = 2,31%), *Escherichia/Shigella* (P = 4,52% vs R = 5,14% vs C = 2,14%), and *Streptococcus salivarius* (P = 1,17% vs R = 0,31% C = 0,50%).

Table 6. Significant differences in bacterial taxa abundances in fecal samples of the three experimental groups.

Taxon	Relative abundance (%)			Comparisons producing p value < 0,05 (t-test)
	PATIENTS	RELATIVES	CONTROLS	
Phylum				
Bacteroidetes	21,16%	17,40%	10,80%	CFS PATIENTS vs CONTROLS (0,0105)
Class				
Phylum Bacteroidetes				
Bacteroidia	20,70%	17,12%	8,63%	CFS PATIENTS vs CONTROLS (0,0005) RELATIVES vs CONTROLS (0,0157)
Phylum Firmicutes				
Clostridia	64,28%	67,79%	76,96%	CFS PATIENTS vs CONTROLS (0,0239)
Order				
Phylum: Firmicutes				
Clostridiales	64,26%	67,77%	76,95%	CFS PATIENTS vs CONTROLS (0,0238)
Phylum Bacteroidetes				
Bacteroidales	20,70%	17,12%	8,63%	CFS PATIENTS vs CONTROLS (0,0005) RELATIVES vs CONTROLS (0,0157)
Family				
Phylum: Firmicutes				
Lachnospiraceae	18,07%	20,56%	30,11%	CFS PATIENTS vs CONTROLS (0,0345)
Phylum: Bacteroidetes				
Bacteroidaceae	9,93%	6,59%	1,27%	CFS PATIENTS vs CONTROLS (0,0002) RELATIVES vs CONTROLS (0,0030)
Barnesiellaceae	1,71%	1,15%	0,27%	CFS PATIENTS vs CONTROLS (0,0005) RELATIVES vs CONTROLS (0,0089)
Genus				
Phylum: Bacteroidetes	Fam:	Bacteroidaceae		
Bacteroides	9,93%	6,59%	1,27%	CFS PATIENTS vs CONTROLS (0,0002) RELATIVES vs CONTROLS (0,0030)
Phylum: Firmicutes	Fam:	Lachnospiraceae		
Anaerostipes	0,18%	0,47%	1,15%	CFS PATIENTS vs CONTROLS (0,0220) RELATIVES vs CONTROLS (0,0326)
Phylum: Firmicutes	Fam:	Veillonellaceae		
Phascolarctobacterium	1,17%	0,76%	0,15%	CFS PATIENTS vs CONTROLS (0,0043)
Phylum: Firmicutes	Fam:	Ruminococcaceae		
Ruminococcus	6,60%	11,48%	8,21%	CFS PATIENTS vs RELATIVES (0,0194)
Species				
Phylum: Bacteroidetes	Fam:	Bacteroidaceae		
Bacteroides uniformis	1,23%	0,91%	0,14%	CFS PATIENTS vs CONTROLS (0,00005) RELATIVES vs CONTROLS (0,0169)
Bacteroides ovatus	0,98%	1,01%	0,10%	CFS PATIENTS vs CONTROLS (0,0001) RELATIVES vs CONTROLS (0,0021)
Phylum: Firmicutes	Fam:	Ruminococcaceae		
Ruminococcus bromii	2,25%	4,89%	1,89%	RELATIVES vs CONTROLS (0,0314)

4.2.2 Oral microbiota diversity

As for intestinal bacterial communities, α-diversity indices were estimated for each analyzed subject. Results showed no differences regarding the total Observed species, the Chao1 and Simpson's diversity indices among CFS/ME patients, their relatives and the external control group. (Table 7)

Table 7. OTU-based diversity indexes in salivary samples of the experimental groups of subjects. *The table shows the total number of OTUs, (b) Chao index and (c) Simpson's evenness indexes in CFS patients, patients' relatives and external control group respectively. Univariate (ANOVA) test is applied in order to assess significant differences between groups (P < 0.05).*

α-diversity indices	**P** (mean)	**R** (mean)	**C** (mean)	**P- value (ANOVA)**
Chao1	9522,56 ± 1521,59	9793,04 ± 2118,92	9347,01 ± 2217,91	0,65
Sobs	1257,96 ± 97,14	1216,97 ± 91,50	1235,77 ± 139,43	0,37
Simpson	27,02 ± 11,06	27,36 ± 14,48	28,41 ± 15,27	0,91

The distances between the oral microbiome of all participants was assessed by Principal Component Analysis (PCA) and by Canonical Correspondence Analysis (CCA).

PCA results showed a full overlapping of CFS patients, their relatives and external controls (Fig. 20).

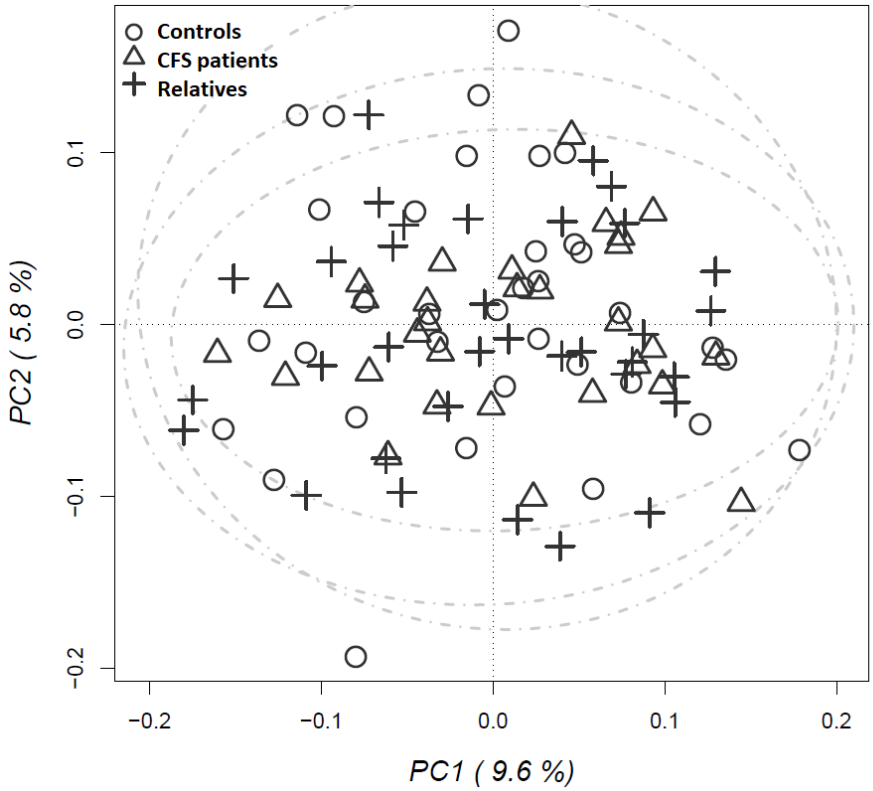

Figure 20. Principal component analysis (PCA) of salivary microbiota compositions in the experimental groups of subjects. *PCA was performed based on abundance of classified OTUs in CFS patients, patients' relatives and external healthy controls, respectively. The percentages on each axis indicate the variation in the samples. Individuals are labeled according to the three groups studied.*

Multivariate CCA was carried out on OTUs abundance table in order to evaluate if the microbial community distribution across individuals was affected by the health state. In the Fig. 21 CCA results were illustrated. Despite CCA showed a clear discrimination between samples, with

CFS/ME patients that clustered apart from the other two groups, the model was not significant (p = 0,379) and explained only 2,1% of the total variance.

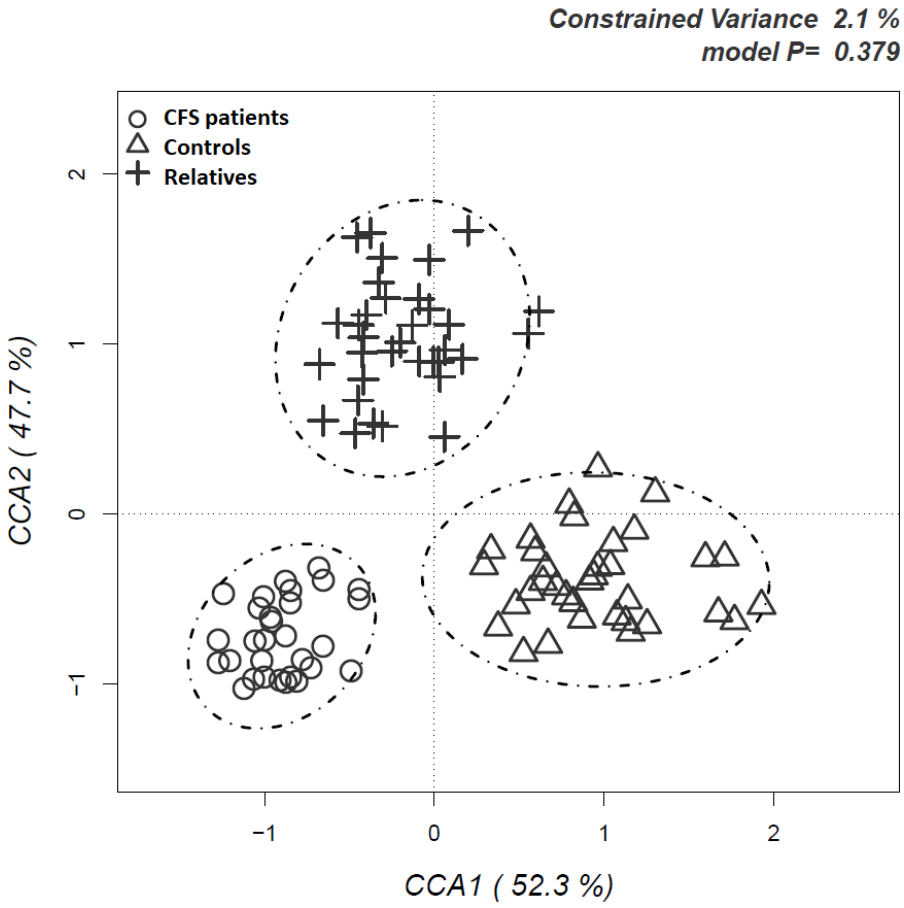

Figure 21. Canonical correspondence analysis (CCA) of salivary microbiota compositions in the experimental groups of subjects. *CCA was performed based on abundance of classified OTUs with frequency >99.9% in CFS patients, patients' relatives and external healthy controls, respectively. The plot shows that the disease status is not a significant source of variability in bacterial communities, explaining only 2.1% of the variance in salivary microbiota.*

When clustering analysis was carried out on OTUs classified at phylum level, a dominance of *Firmicutes*, followed by *Actinobacteria*, *Fusobacteria* and *Proteobacteria* was found (Fig. 22 A). No grouping was observed, highlighting that the oral microbiomes of the three experimental groups were similar.

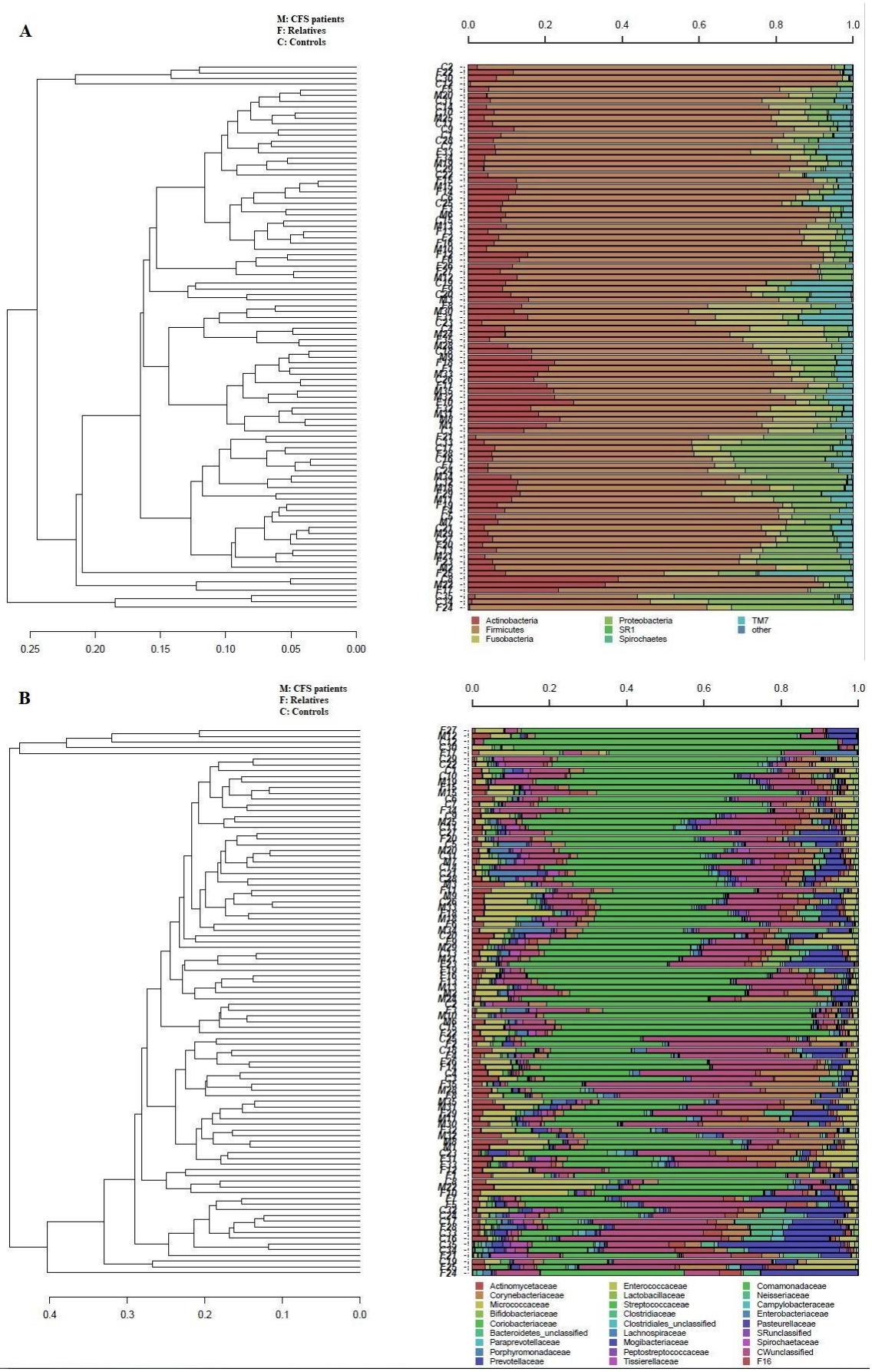

Figure 22. Hierarchical clustering of classified sequences at phylum (A) and family (B) classification levels. *The three experimental groups are indicated as M (CFS patients), F (patients' relatives), and C (external healthy controls). Bars of different colors indicate the relative percentage of bacterial phyla identified in each salivary sample. Only taxa ≥5% in at least one sample are shown. Similar samples were clustered using the average linkage algorithm.*

At family level, *Streptococcaceae* was the dominant family, followed by *Actinomycetaceae*, *Micrococcaceae* and *Pausterellaceae* (Fig. 22 B). As observed at phylum level, no evident clustering was observed.

In order to detect OTUs whose relative presence was significantly different between groups, Metastats analysis was carried out for salivary samples. Six out of the 30 most abundant OTUs had relative abundance ≥ 4% and included *Streptococcus infantis* (OTU00002), *Veillonella parvula* (OTU00003), *unclassified TM7-3* (OTU00006) (Fig. 23). The OTUs classified as *Rothia dentocariosa* (OTU00011) and *Neisseria subflava* (OTU00025) showed significant differences among experimental groups. In particular, CFS/ME patients and their relatives had a higher relative aboundance of *Rothia dentocariosa* compared to external controls, while *Neisseria subflava* was significantly lower only in patients' relatives.

Figure 23. Relative abundance of specific OTUs across the three experimental groups. *Metastats model was applied on the 30 most abundant OTUs. Significant differences are highlighted by different minor letters (P < 0.05).*

As for fecal samples, the identified OTUs in oral microbiota were classified at different taxonomic levels in order to determine the relative abundance of different bacterial taxa between samples. The significant differences between experimental groups in the relative abundances of oral bacterial taxa are reported in Table 8. The oral cavity bacteria grouped within six main bacterial phyla: *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Bacteroidetes* and *TM7* (Fig. 24). The predominant phylum *Firmicutes* (P= 64,20% vs R = 65, 02% vs C = 64,75%), and *Bacteroidetes* (P = 4.26% vs R = 4.02% vs C = 5.17%) did not differ in terms of relative abundances among the three experimental groups. *Actinobacteria* (P = 12,29% vs R = 10,47% vs C = 7,92%) were more abundant in CFS/ME patients (p = 0,01) compared to external controls. Furthermore, a decreased proportion of *Proteobacteria* (P = 6,97% vs R = 8,77% vs C = 9,85%) and *TM7* (P = 3,72% vs R = 4,25% vs C = 4,91%) and a higher abundance of *Fusobacteria* (P = 8,28% vs R = 7,24% vs C = 6,78%) were also detected in CFS/ME subjects and in their relatives.

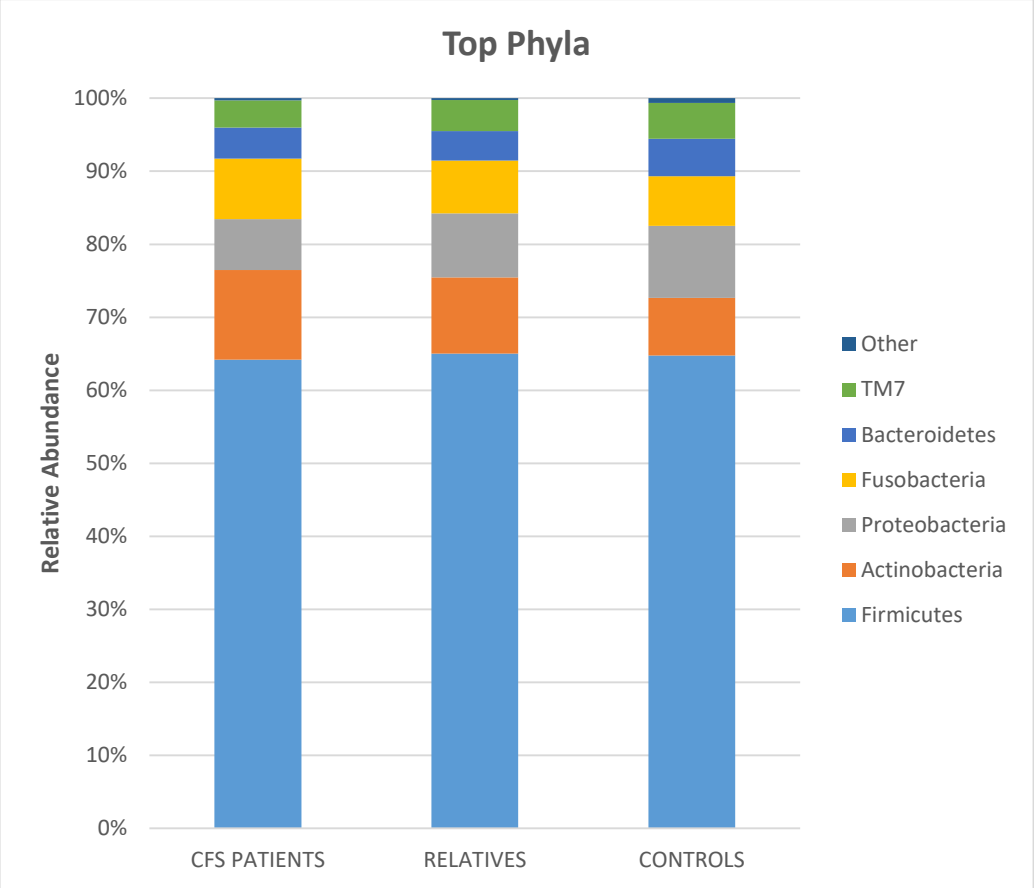

Figure 24. Compositions of the oral microbiota from CFS patients, relatives and controls at phylum level. *Only taxa ≥1% in at least one sample are shown, while taxa with lower participations were added to the "other" sequence group.*

The *Firmicutes*/*Bacteroidetes* ratio (P = 15,08 ± 4,06 vs R = 16,18 ± 3,87 vs C = 12,52 ± 3,11) did not differ among groups (Fig. 25).

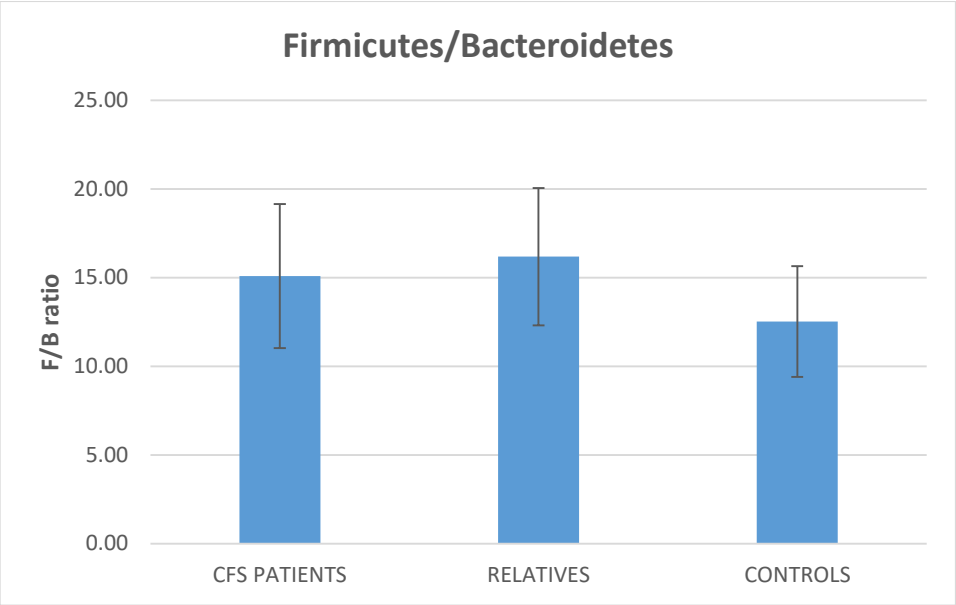

Figure 25. Firmicutes/Bacteroidetes (F/B) ratio in salivary samples across the three experimental groups. *Comparison of F/B ratio between CFS patients, their relatives and external controls.*

A total of 51 orders were identified in the oral microbiota of the three groups. Ten orders were found to have a relative aboundance ≥ 1% in at least one sample (Fig. 26).

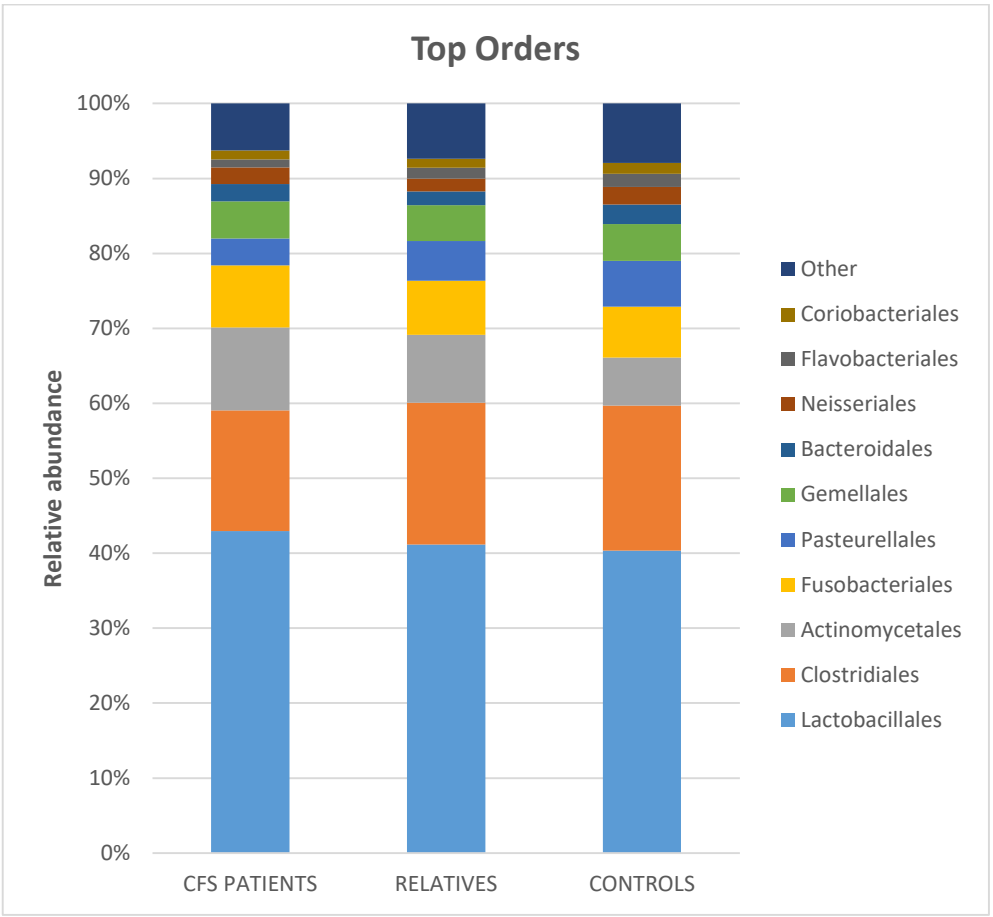

Figure 26. Compositions of the oral microbiota from CFS patients, relatives and controls at order level. *Only taxa ≥1% in at least one sample are shown, while taxa with lower participations were added to the "other" sequence group.*

The most interesting variations were observed for *Actinomycetales* (P = 11,05% vs R = 9,03% vs C = 6,38%), significantly higher in CFS/ME patients (p = 0,005) compared to external controls, and *Pasteurellales* (P = 3,58% vs R = 5,28% vs C = 6,12%). *Clostridiales* (P = 16,09% vs R = 18,92% vs C =19,38%) and *Flavobacteriales* (P = 1,08% vs R = 1,41% vs C = 1,75%) resulted less abundant in CFS patients and in their relatives; conversely, *Fusobacteriales* (P = 8,28% vs R = 7,24% vs C = 6,78%) was more abundant in CFS/ME patients and their relatives (not statistically significant).

The most aboundant families were *Streptococcaceae* (P = 40,19% vs R = 38,86% vs C = 38,30%) and *Veillonellaceae*, followed by *Leptotrichiaceae*, *Pasteurellaceae*, *Gemellaceae*, *Actinomycetaceae*, *Neisseriaceae*, *Carnobacteriaceae*, *Fusobacteriaceae* and *Flavobacteriaceae*. (Fig. 27).

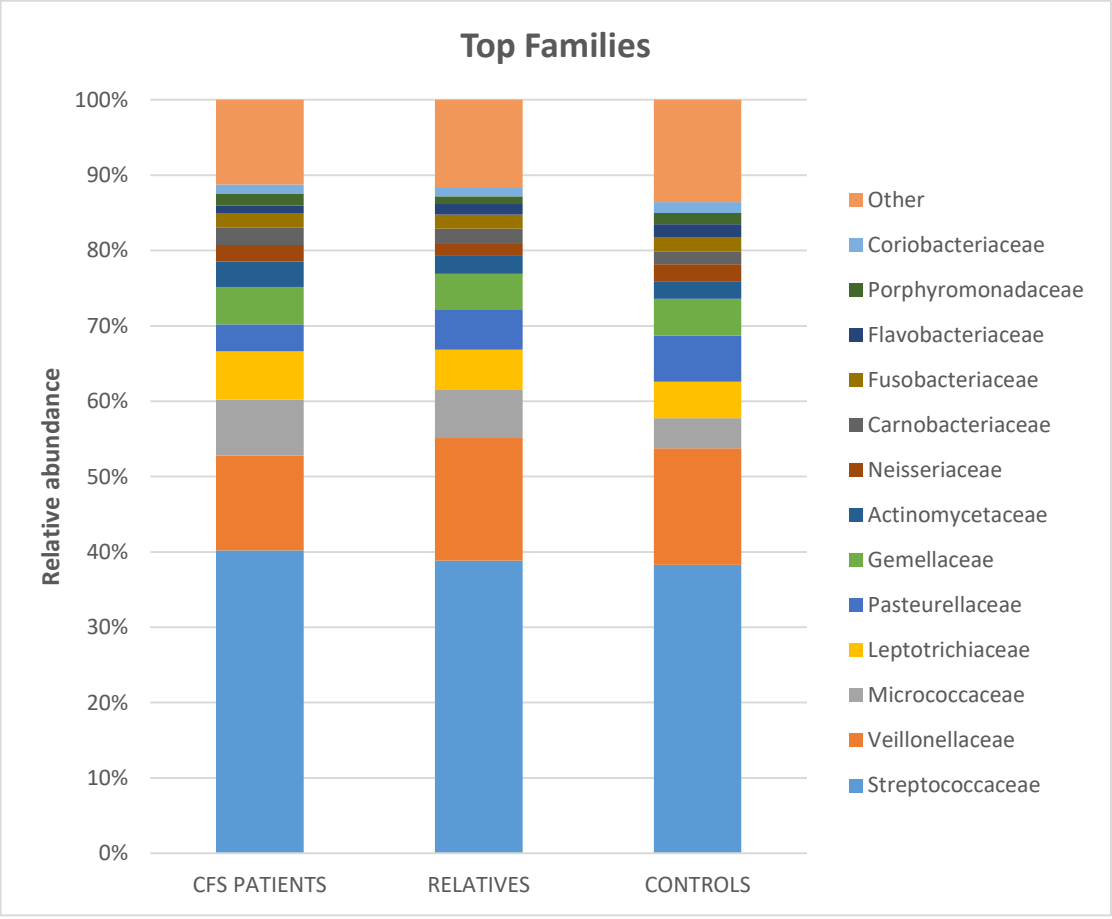

Figure 27. Compositions of the oral microbiota from CFS patients, relatives and controls at family level. *Only taxa ≥1% in at least one sample are shown, while taxa with lower participations were added to the "other" sequence group.*

The greatest variations were observed for two families belonging to *Actinomycetales* order, *Micrococcaceae* (P = 7,44% vs R = 6,39% vs C = 3,98%), significantly higher in CFS/ME

patients (p = 0.014), and *Actinomycetaceae* (P = 3,37% vs R = 2,39% vs C = 2,22%) that were more abundant in CFS/ME patients. *Pasteurellaceae* (P = 3,58% vs R = 5,28% vs C = 6,12%), *Veillonellaceae* (P = 12,59% vs R = 16,25% vs C = 15,45%) and *Flavobacteriaceae* (P = 1,04% vs R = 1,39% vs C = 1,72%) were less frequent in CFS/ME patients, while the opposite was recorded for *Leptotrichiaceae* (P = 6,40% vs R = 5,35% vs C = 4,86%) and *Carnobacteriaceae* (P= 2,27% vs R = 1,89% vs C = 1,70%).

Streptococcus, *Veillonella*, *Rothia*, *Leptotrichia*, *Haemophilus*, *Gemella*, *Actinomyces*, *Granulicatella*, *Neisseria*, *Fusobacterium*, *Capnocytophaga* and *Porphyromonas* were the most representative genera, with a relative abundance greater than 1% (Fig. 28). In particular, CFS/ME patients exhibited differences in the relative abundances of members belonging to *Actinomycetales* order.

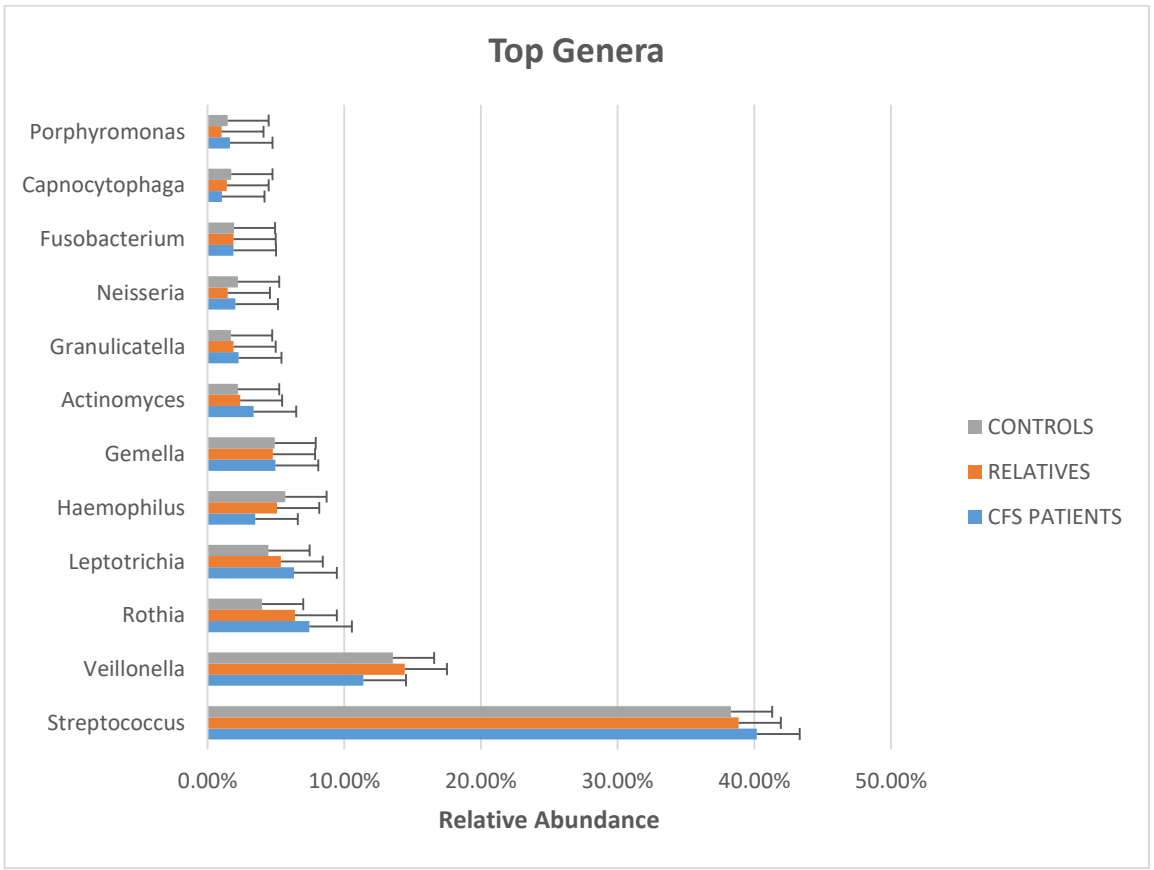

Figure 28. Compositions of the oral microbiota from CFS patients, relatives and controls at genus level. *Only taxa ≥1% in at least one sample are shown.*

The genus *Rothia* (P = 7,44% vs R= 6,38% vs C = 3,97%) was significantly more abundant in CFS/ME patients (p = 0.014). *Actinomyces* (P = 3,36% vs R = 2,38% vs C = 2,21%), *Leptotrichia* (P = 6,32% vs R = 5,35% vs C = 4,44%) and *Granulicatella* (P = 2,27% vs R = 1,89% vs C = 1,70%) showed higher abundance in CFS/ME patients, while the opposite was

recorded for *Haemophilus* (P = 3,47% vs R = 5,08% vs C = 5,69%), *Capnocytophaga* (P = 1,04% vs R = 1,39% vs C = 1,72%) and *Veillonella* (P = 11,39% vs R = 14,42% vs C = 13,55%). At species level (Fig. 29), the most evident differences were observed for the species *Rothia mucilaginosa* (P = 5,74% vs R = 4,37% vs C = 3,18%) and *Rothia dentocariosa* (P = 1,03% vs R = 1,53% vs C = 0,50%), both members belonging to Actinobacteria phylum, with the latter significantly increased both in CFS/ME patients (p = 0,01) and in their relatives (p = 0.010) compared to external control group.

Streptococcus infantis (P = 27,63% vs R = 26,39% C =26,38%) was the most abundant species in all groups. *Neisseria subflava* (P = 1,92% vs R = 1,22% vs C = 2,11%), a member belonging to *Proteobacteria*, was significantly less abundant only in patients' relatives (p = 0,028), while no substantial differences were observed between CFS/ME patients and external controls. *Haemophilus parainfluenzae* (P = 3,41% vs R = 4,23% vs C = 5,45%), *Streptococcus salivarius* (P = 2,48% vs R = 3,14% vs C = 4,24%) and *Veillonella dispar* (P = 6,20% vs R = 9,38% vs C = 7,91%) showed a decreased abundance in CFS/ME patients.

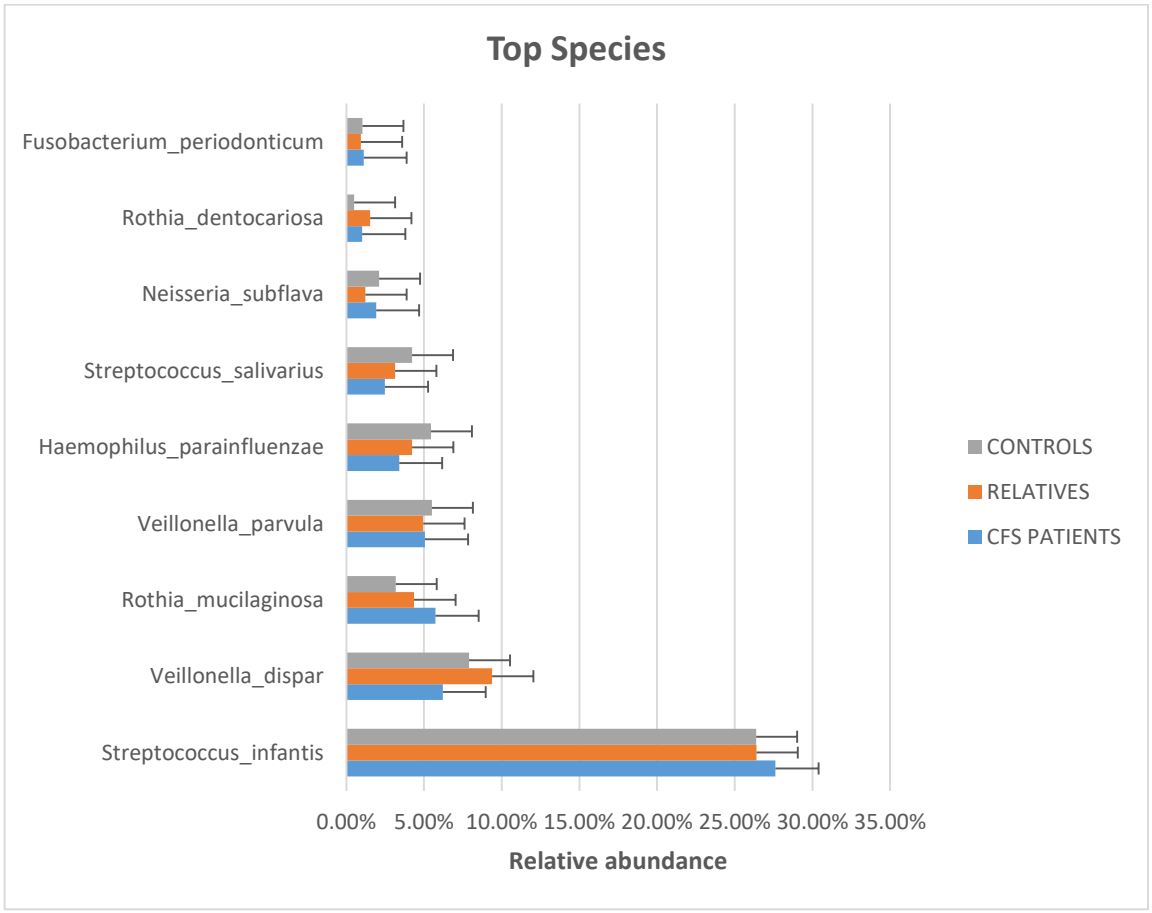

Figure 29. Compositions of the oral microbiota from CFS patients, relatives and controls at species level. *Only taxa ≥1% in at least one sample are shown.*

Table 8. Significant differences in bacterial taxa abundances in salivary samples of the three experimental groups.

Taxon	Relative abundance (%)			Comparisons producing p value < 0,05 (t test)
	PATIENTS	RELATIVES	CONTROLS	
Phylum				
Actinobacteria	12,29%	10,47%	7,92%	CFS PATIENTS vs CONTROLS (0,014)
Order				
Actinomycetales	11,05%	9,03%	6,38%	CFS PATIENTS vs CONTROLS (0,005)
Family				
Phylum: Actinobacteria				
Micrococcaceae	7,44%	6,39%	3,98%	CFS PATIENTS vs CONTROLS (0,014)
Genus				
Phylum: Actinobacteria	Fam:	Micrococcaceae		
Rothia	7,44%	6,38%	3,97%	CFS PATIENTS vs CONTROLS (0,014)
Species				
Phylum: Actinobacteria	Fam:	Micrococcaceae		
Rothia dentocariosa	1,03%	1,53%	0,50%	CFS PATIENTS vs CONTROLS (0,034) RELATIVES vs CONTROLS (0,010)
Phylum: Proteobacteria	Fam:	Neisseriaceae		
Neisseria subflava	1,92%	1,22%	2,11%	RELATIVES vs CONTROLS (0,028)

4.3 UPLC-MS analysis

Based on the results of the metagenomic analysis of intestinal microbiota in CFS/ME patients, to evaluate whether the metabolic profile of CFS/ME patients differed from those of their relatives and controls, a pilot study was conducted on the fecal samples of 5 CFS/ME patients (M12-M2-M10-M7-M24) which belonged to a same cluster at family level. Five relatives of these patients and five external controls were also selected. The UPLC-MS analysis was carried out according to a untarget approach and resulted in 4848 extracted features. These features, with their relative retention time and single mass spectrum, were normalized for the baseline and the percentile shift at 75%, and filtered in order to eliminate features that were potentially not interesting for analysis.

PCA was applied to explore the distribution of the samples and evaluate the presence of clusters (Fig. 30).

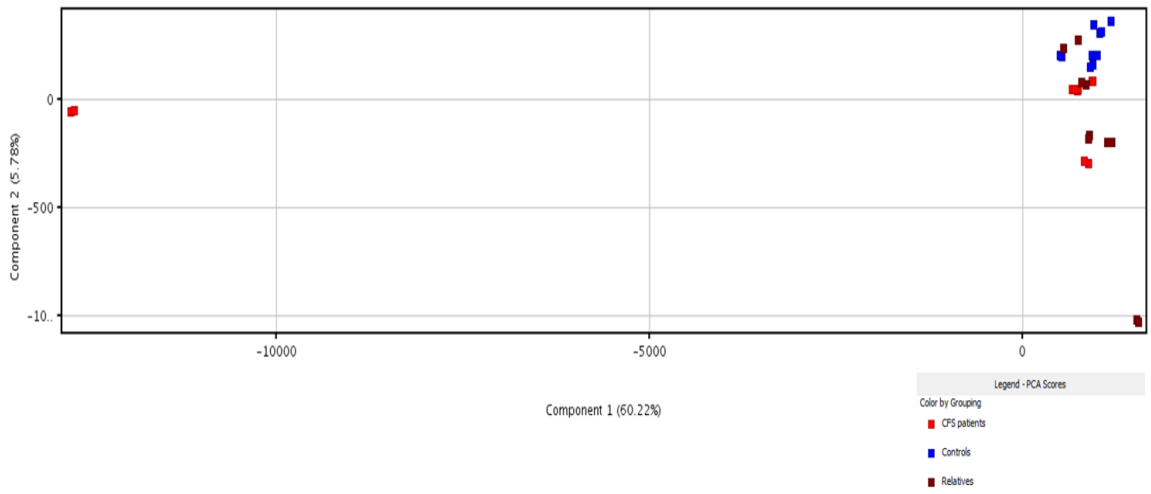

Figure 30. PCA (unsupervised) model for the classification of the three experimental sub-groups. *In red are represented CFS patients, in brown the relatives and in blue the controls.*

The three groups of subjects under investigation were distinct, with a total explained variability > 65%, although a clear separation was not observed and except for one sample.

Partial Least Square-Discriminant Analysis (PLS-DA) showed a more evident separation between CFS/ME patients and healthy subjects (Fig. 31).

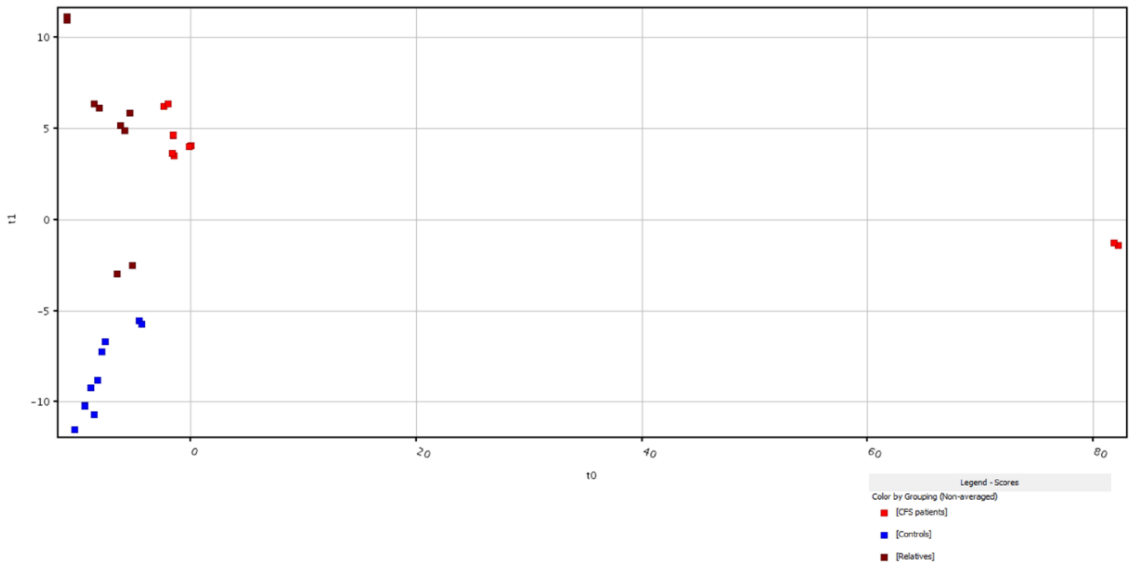

Figure 31. PLS-DA (supervised) model through multivariate statistical analysis with the representation of the two principal components for the classification of the three experimental groups. In red are represented CFS patients, in brown the relatives and in blue the controls.

The filtered data set was used for an Orthogonal Latent Structure-Discriminant Analysis (OPLS-DA) classification model using Pareto scaling. The application of the OPLS-DA analysis allowed to discriminate CFS/ME patients from their relatives and external controls (Fig. 32).

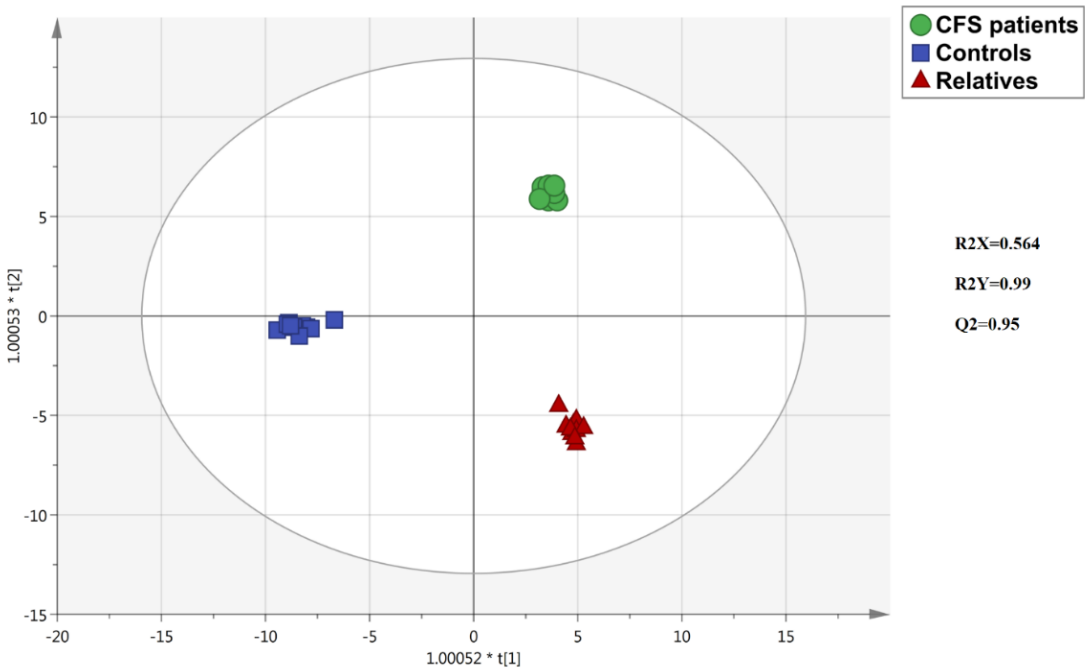

Figure 32. OPLS-DA (supervised) model for the classification of the three experimental sub-groups. *R2 indicates the % variability explained by the model; Q2 estimates the predective power of the model.*

The permutation test was performed in order to show that the differences between the groups, highlighted by the OPLS-DA analysis, are not the result of random associations between features. The results showed that the model is not random (Q2<0, Fig. 33).

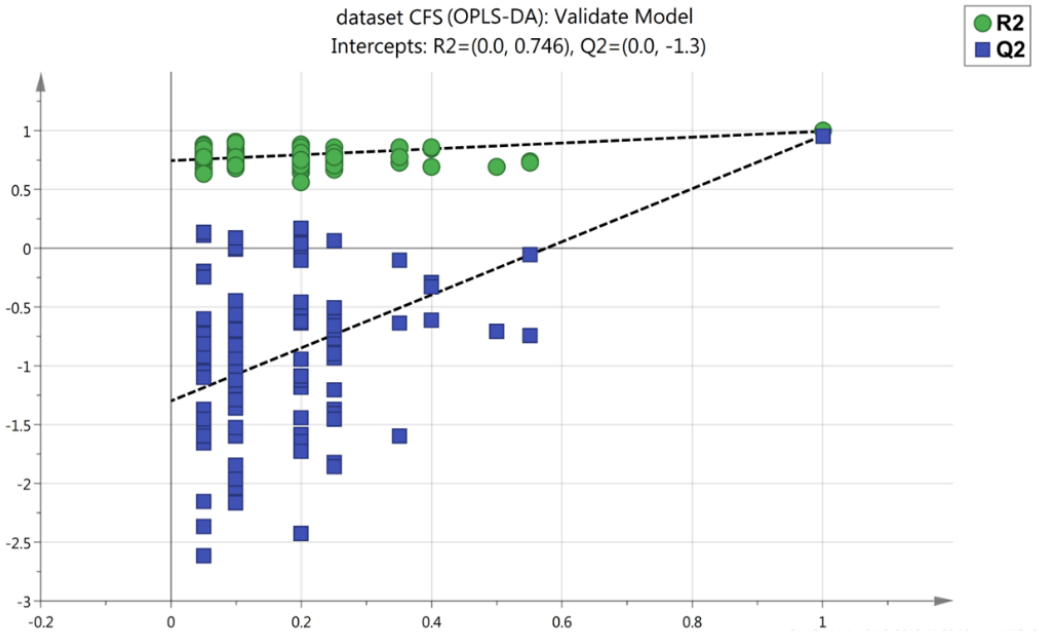

Figure 33. Permutation test for the three experimental sub-groups. *R2 indicates the % variability explained by the model; Q2 estimates the predictive power of the model.*

Using MS-DIAL software, 1403 single-mass compounds were identified by comparing the retention time and mass spectrum of the features to those reported in MoNA database. Of these, 125 metabolites also had a MS/MS spectrum, but only 7 matched 100% after alignment. An in silico prediction was performed aligning the characteristics of retention time and mass spectrum of the features to those in other databases (i.e Human Metabolome Database) and determining an affinity score.

Using the MS Finder software, a multivariate OPLS-DA analysis was performed resulting in a visual separation of annotated metabolomes in the two-dimensional space (Fig. 34).

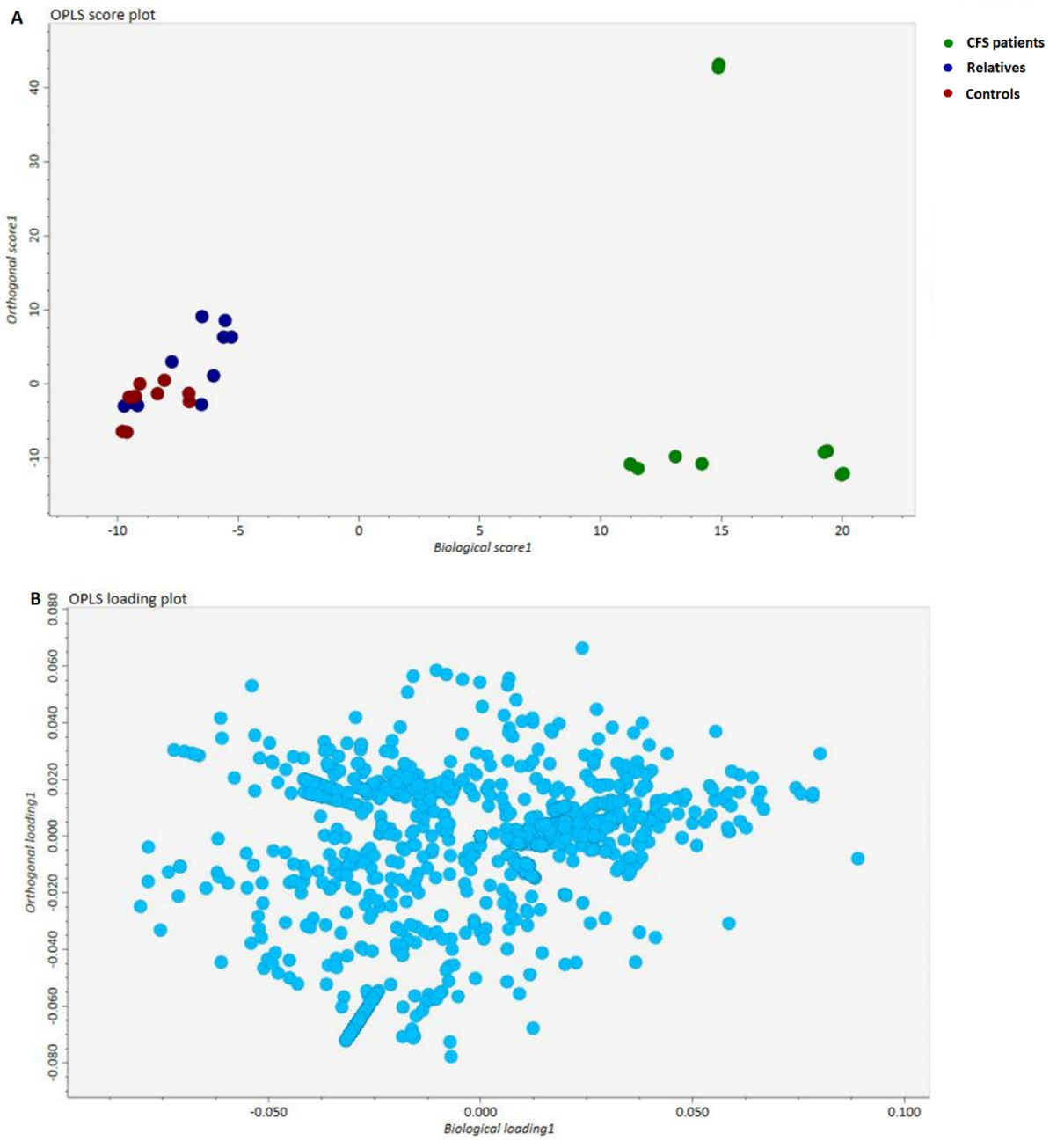

Figure 34. The score (A) and loading (B) plots from OPLS-DA model. In the loading plot, the variables which contributes the most to group discrimination are shown in blue.

Results clearly showed that the metabolic profile of CFS/ME patients is quite different from that of their relatives and external controls. In the *score* plot, biological variability explained the differences observed between CFS/ME patients and healthy subjects (relatives and external controls), while orthogonal variability explained the differences between patients' relatives and external controls. Interestingly, the patients' relatives and controls tended to cluster together, showing that their metabolic profiles were similar.

VIP (Variable Importance in Projection) scores, a measure of a variable's contribution to the OPLS-DA model, were calculated for all 1403 identified compounds. Of these, 559 compounds

showed a VIP score greater than 1 and represented the most contributory variables in group discrimination in the OPLS-DA model.

Several compounds derived from bacterial metabolism, such as SCFAs (acetate, propionate and butyrate), branched-chain fatty acids (BCFA: isobutyrate, valerate, and isovalerate), indole derivatives, choline metabolites, mono- and polyamines, amino acids, peptides, vitamins and bile acid metabolites, contributed to the metabolic differentiation between the three experimental groups (Table 9).

Table 9. Annotated metabolites derived from bacterial metabolism. *Metabolites which differed between CFS patients (P), their relatives (R) and external controls (C) and with VIP (Variable Importance in Projection) scores > 1 as assessed by OPLS-DA model. Fold changes of metabolites were the ratio of the average MS ion intensities (peak areas) in CFS patients, their relatives and external controls.*

ID	Metabolite name	Average Rt (min)	Average Mz	F-change P/C	p value	F-change R/C	p value	VIP score
	Butyric acid							
3673	-4-oxobutanoic acid	9,1	545,3	12,8	0,28	4,2	1,0	2,3
1300	-butanoic acid	2,6	269,2	0,0	0,40	0,1	0,55	1,6
463	4-acetamidobutanoate	1,8	146,1	3,2	0,83	1,0	1,0	1,3
3730	-2-ethylbutanoate	13,8	550,3	4,4	0,20	1,0	0,38	1,2
3152	-4-oxobutanoic acid	10,8	483,3	2,8	0,20	1,0	0,38	1,2
3429	-butanoate	12,3	517,1	2,9	0,18	1,0	0,22	1,2
2825	-2-methylidenebutanoate	9,7	444,2	2,8	1,0	1,0	1,0	1,2
2680	-4-oxobutanoic acid	9,7	426,3	0,5	1,0	1,7	1,0	1,1
3419	-butanoic acid	5,1	516,2	0,1	1,0	0,3	1,0	1,1
	Propionic acid							
5248	-propanoic acid	11,3	807,6	0,4	0,11	0,9	1,0	1,6
3444	-propanoic acid	12,4	519,1	2,8	0,52	1,0	1,0	1,3
4038	-propanoic acid	12,9	582,4	2,8	0,18	1,0	0,22	1,2
4148	-2-methylpropanoic acid	12,4	591,3	0,2	0,64	0,6	1,0	1,2
5107	-propanoic acid	13,0	738,2	8,6	0,30	1,0	0,51	1,1
3738	-3-oxopropanoic acid	11,4	551,1	987,7	1,0	1,0	1,0	1,0
3252	-2-methylpropanoate	7,2	496,3	1,0	1,0	7,7	1,0	1,0
	Acetic acid							
3002	-acetate	8,7	466,3	5,0	1,0	0,0	1,0	2,0
1520	-acetate	5,6	295,1	27,4	0,26	12,2	0,62	1,5
3615	-acetate	8,9	539,2	3,7	0,86	0,3	1,0	1,4
829	-acetic acid	2,9	192,1	2,8	1,0	2,8	0,85	1,4
4456	-2-phenylacetate	14,6	616,2	11,4	1,0	1,0	1,0	1,2
3508	-acetate	11,3	527,3	2,8	1,0	1,0	1,0	1,2
5084	-acetate	11,4	728,5	2,8	1,0	1,0	1,0	1,1
2344	-acetate	11,2	375,3	0,4	0,10	1,1	1,0	1,1
3302	-diacetate	8,6	501,2	2,8	0,40	1,0	0,14	1,0
	Isobutyric acid							
891	-2-methylpropanoic acid	2,5	204,1	0,8	1,0	0,7	1,0	1,1
	Valeric acid							

1716	-3-methylpentanoate	8,3	310,2	4,6	0,13	3,2	0,82	2,2
2713	-3-methylpentanoic acid	12,6	429,3	0,1	0,39	1,5	1,0	1,1
	Isovaleric acid							
3445	-3-methylbutanoic acid	9,6	519,3	2,8	1,0	1,0	1,0	1,2
	Mono- and polyamines							
539	Dopamine	1,8	154	1,8	0,84	0,7	1,0	2,9
540	N-Acetylhistamine	1,8	154,1	0,0	0,64	0,0	0,68	2,0
809	N8-Acetylspermidine	1,6	188,2	0,2	0,21	0,2	0,28	1,5
356	Phenylethanolamine	2,1	138,1	1,2	1,0	0,7	1,0	1,5
2886	-3-phosphoethanolamine	13,8	454,3	0,0	0,42	0,2	0,75	1,3
469	Spermidine	1,6	146,2	0,7	1,0	0,9	1,0	1,3
678	3-Methoxytyramine	2,3	168,1	3,0	1,0	1,0	1,0	1,2
630	N-acetyl-2-phenylethylamine	2,8	164,1	0,9	1,0	0,1	0,65	1,0
299	N-Acetylputrescine	1,6	131,0	2,8	1,0	1,0	1,0	1,2
	Indole derivatives							
3012	-isoindole-6'-one	9,2	468,2	6,0	0,10	1,0	0,40	2,1
3062	-isoindole-1,15-dione	7,0	472,2	0,1	0,42	1,0	1,0	1,9
2847	-isoindole-6'-one	16,8	447,3	8,8	0,53	3,0	1,0	1,4
4398	-indole-3-carboxylic acid	14,6	613,2	3,3	0,35	1,0	0,29	1,3
2754	-indole-2,5(3H)-dione	2,0	434,2	0,0	0,34	0,2	0,61	1,3
946	-indole	3,3	213,1	2,8	1,0	1,0	1,0	1,2
1567	-indole-1,4(3H,5aH)-dione	5,9	298,2	2,8	1,0	1,0	0,45	1,2
798	-indole	3,5	187,1	2,8	0,33	1,0	0,76	1,2
2792	-isoindole-1,15(2H)-dione	13,1	440,3	0,3	0,35	0,4	0,37	1,2
2999	-isoindole-1,15(2H)-dione	9,0	466,3	2,8	0,30	1,0	0,51	1,1
2640	-isoindole-1,15(2H)-dione	8,5	420,3	2,8	1,0	1,0	1,0	1,1
2763	-isoindole-2,16-dione	8,7	435,3	2,8	0,61	1,0	0,51	1,1
288	Indole-3-carbinol	2,6	130,1	2,8	1,0	1,0	1,0	1,1
3000	-isoindole-1,16(2H)-dione	6,8	466,3	0,1	1,0	0,2	1,0	1,1
600	N-Acetylindole	2,3	160,1	0,3	1,0	0,7	1,0	1,0
	Choline metabolites							
917	Benzoylcholine	2,8	208,1	4,8	0,41	1,7	1,0	1,6
464	Acetylcholine	2,7	146,1	0,1	0,18	0,1	0,22	1,2
136	Betaine	1,8	118,1	0,1	1,0	0,8	1,0	1,1
	Vitamins							
985	Pantothenic acid (B5)	2,0	220,1	1,5	1,0	3,4	1,0	2,0
	Bile acid metabolites							
2604	Alpha-hydroxydeoxycholic acid	11,2	415,3	0,1	0,36	2,6	1,0	1,4
	Amino acids							
812	N-alpha-acetyl-L-lysine	1,9	189,1	0,2	0,38	0,5	1,0	2,1
134	Norvaline	13,1	118,1	0,3	1,0	3,2	0,64	1,3
651	Phenylalanine	2,0	166,1	0,1	0,2	1,1	1,0	1,3
139	beta-N-Methylaminoalanine	3,7	119,1	2,9	0,18	1,0	0,22	1,2
491	L-Glutamate	2,1	148,1	0,2	0,8	0,6	1,0	1,0
	Peptides							
547	Cyclo-prolylglycine	1,9	155,1	0,8	1,0	0,8	1,0	1,9
1241	Prolylphenylalanine	3,0	263,1	0,3	0,06	0,4	0,26	1,8

An overall increase of SCFAs derivatives was observed in fecal samples of CFS/ME patients, while no difference was recorded between their relatives and external controls, except for some metabolites (not statistically significant). Interestingly, -3-oxopropanoic acid (ID3738) showed a very high level in CFS/ME patients compared to external controls, while their relatives did not differ. Moreover, CFS/ME patients and their relatives reported high levels of -4 oxobutanoic acid (ID3673), acetate (ID1520) and -3-methylpentanoate (ID1716). An increase of -3-methylpentanoate (a valerate derivative) was observed both in CFS/ME patients and in their relatives compared to controls, while no difference in isobutyrate level was observed among the three populations. In addition, CFS/ME patients showed high level of isovalerate, not observed in their relatives. All amino acids levels (except for beta-N-Methylaminoalanine) were slightly decreased in CFS/ME patients compared to external controls. Three biogenic amines derived from amino acids metabolism, such as dopamine (ID539), -3-methoxytyramine (ID678), N-acetilputrescine (ID299), and several indole derivatives were increased only in CFS/ME patients (not statistically significant).

5. DISCUSSION

In recent years, several studies have established the important role played by the microbiota in human health and its implication in disease development. (Robinson *et al.*, 2010; Bassis *et al.*, 2013). In a state of equilibrium, which can be identified with the state of health, there is a functional cooperation between the host and colonizers that enhances nutrient metabolism, the synthesis of vitamins, containment of pathogen proliferation and development and maturation of the immune system (Nicholson *et al.*, 2012; Kau *et al.*, 2011). The oral and intestinal microbiota represent the most dominant microbial communities in humans, and it is well known that both are able to display signatures associated with pathologies (Abe *et al.*, 2018; Shengtao *et al.*, 2019).

As concerns the CFS/ME syndrome, which is characterized by chronic fatigue and multisystemic alterations, till now the relationship between the bacterial composition and pathogenesis of CFS/ME has not been fully demonstrated, although previous studies reported alterations of intestinal and oral microbiota (Fremont *et al.*, 2012; Shukla *et al.*, 2015; Giloteaux *et al.*, 2016; Nagy-Szakal *et al.*, 2017; Wang *et al.*, 2018). Some features of this syndrome, such as the "*relapsing-remitting*" symptoms and persistent immune activation, suggest a similarity with autoimmune conditions. Another feature that deserves to be emphasized, is the cognitive impairment frequently observed in CFS/ME patients (Chen, *et al.*, 2008). As a consequence, the possible disturbance of the so-called microbiota-gut-brain axis needs to be further investigated.

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. However, some differences, although not statistically significant, deserve to be highlighted.

First of all, the intestinal and oral microbiota of CFS/ME patients exhibited modifications in the bacterial composition compared to healthy controls, due to changes in the relative abundances of several bacterial taxa. Interestingly, the relatives, in the most cases, showed intermediate prevalence values.

β-diversity analyses showed a reduced inter-individual variability between CFS/ME patients and healthy subjects for both the salivary and fecal microbiota.

Considering the fecal microbiota, the analysis at taxon level showed a reduction of *Firmicutes* (F) and, on the contrary, a significant increase of *Bacteroidetes* (B) in CFS/ME patients in

comparison with the non-CFS/ME groups. The prevalence of these phyla showed intermediate values in patients' relatives, suggesting an environmental influence. A further evidence of environmental conditioning is the observation of a similar increased prevalence of *Proteobacteria* in patients and in their relatives. Considering the F/B ratio, a progressive decrease of this index was observed in CFS/ME patients and in their relatives, with the highest value in external controls and the lowest in patients.

These results agree with those of previous studies, reporting that the intestinal microbiome in CFS/ME patients is characterized by less members belonging to *Firmicutes* phylum and more *Bacteroidetes* (Fremont *et al.*, 2012; Shukla *et al.*, 2015; Giloteaux *et al.*, 2016). A similar pattern has been observed also for the fecal microbiome of patients with Alzheimer's disease (Vogt *et al.*, 2017). The microbiome plays an important role in gut-brain communication through the gut-brain axis (Zhul *et al.*, 2017), synthesizing a large number of chemical mediators (including neurotransmitters such as dopamine, serotonin and GABA), which, being released into the bloodstream, can reach distant sites, such as the brain, exerting positive or negative effects on the host health (Clarke *et al.*, 2014). Changes in the composition and abundance of intestinal microorganisms may also affect the host response to stress, through the modification of the intestinal-brain physiology (Cryan *et al.*, 2012), affecting cognitive and behavioral functions (Zhul *et al.*, 2017).

The shift in *Firmicutes*/*Bacteroidetes* ratio observed in the present study has been previously described in various autoimmune conditions, such as Chron's disease (Manichanh *et al.*, 2006) and Systemic Lupus Erythematous (Hevia *et al.*, 2014), and in diabetes type 2 (Larsen *et al.*, 2010). Conversely, an increase in F/B ratio has been observed in relation to obesity (Koliada *et al.*, 2017; Ismail *et al.*, 2011).

In our study, 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. *Lachnospiraceae* are abundant in the gastrointestinal tract of humans (Gosalbes *et al.*, 2011; Rinninella *et al.*, 2019). All members belonging to this family are strictly anaerobic and spore-forming bacteria, and several species are important butyrate producers (Duncan *et al.*, 2002). The capability of producing butyric acid and its important role for both microbial and host epithelial cell growth link the members of this family to both obesity (Duncan *et al.*, 2008; Turnbaugh *et al.*, 2008) and protection from colon cancer in humans (Mandal *et al.*, 2001). A low abundance of *Lachnospiraceae* has been observed in pediatric patients with ulcerative colitis and Chron's disease (Maukonen *et al.*, 2015), both autoimmune conditions. Moreover, several members belonging to this family were

also under represented in the gut of adult IBD patients (Frank *et al.*, 2007). Also patients with non-alcoholic fatty liver disease (NAFLD) and with liver cirrhosis show a significant reduction in *Lachnospiraceae* abundance (Wang *et al.*, 2016; Chen *et al.*, 2011). A previous study, carried out on rats with stressed-induced visceral hypersensitivity, reported a decreased abundance of several OTUs related to butyrate-producing *Lachnospiraceae* and a reduced representation of pathways involved in the metabolism of butyrate.

The decrease in *Lachnospiraceae* that we recorded involved the genera *Roseburia*, *Coprococcus*, *Lachnospira* and *Anaerostipes*. All these genera are dominant in the healthy human gut and most of them are butyrate producers; butyric acid is a SCFA which production not only represents an important energy source for other microbes and for the host's enterocytes, but also contributes to the maintenance of the intestinal barrier and protection of the gut epithelium against inflammation (Venegas *et al.*, 2019). In particular, we observed a significant reduction of *Anaerostipes* both in CFS patients and in their relatives. *Anaerostipes* converts lactate to butyrate and has been found to be reduced also in smoking IBD patients (Morgan *et al.*, 2012).

Similarly, *Roseburia* showed a 50% decrease in CFS/ME patients, although this result was not statistically significant. This finding agrees with a previous pubblished study on CFS/ME patients from northern Europe (Fremont *et al.*, 2012). *Roseburia* is the major butyrate producing genus in the human gut and contributes to the health of colon epithelium.

A decrease in the abundance of this genus has been reported also in inflammatory diseases, such as IBD (Machiels *et al.*, 2014; Takahashi *et al.*, 2016), and in early onset reumatoid arthritis (Vaahtovuo *et al.*, 2008). *Lachnospira* has been reported to decline also for other pathological conditions, such as asthma (Stiemsma *et al.*, 2016) and Chron's disease (Wang *et al.*, 2018) in pediatric patients.

Furthermore, we observed that the fecal microbiota of CFS/ME patients was enriched in *Streptococcus*, a group of D-lactic acid producing bacteria previously observed more abundant in a cohort of CFS/ME patients by *Sheedy et al*. (2009).

Within *Bacteroidales* we detected other interesting modifications. A significant increase in *Bacteroidaceae* and *Barnesiellaceae*, particularly *Bacteroides* and *Barnesiella*, was observed both in CFS/ME patients and their relatives. *Bacteroides* is the predominant genus within the lower human intestinal tract. All members belonging to this genus are Gram-negative, obligate anaerobic and non-spore-forming species. These bacteria play a fundamental role in the processing of complex molecules in the host intestine, particularly in carbohydrate fermentation. This activity results in the production of SCFA, mainly propionate and acetate, which are used by the host as energy source (Wexler *et al.*, 2007). *Bacteroides uniformis* is a

butyrate-producing bacteria normally present in the human gut. This species exhibits expanded greater glycolytic capability with respect to other *Bacteroides* species and is able to use dietary or endogenous glycans to produce butyrate (Benítez-Páez *et al.*, 2017). The oral administration of *Bacteroides uniformis* CECT 7771 was found to ameliorate metabolic and immunological dysfunction in mice with high-fat-diet induced obesity (Gauffin *et al.*, 2012).

Studies on the modification of bacterial composition in relation to diet showed that fecal communities cluster into three enterotypes that are strongly associated with long-term diets. Particularly, *Bacteroides* are predominant in subjects who follow a typical western, rich in protein and lipids diet, while *Prevotella* is the predominant genus in humans with a carbohydrate rich diet, especially fibers, (Wu *et al*., 2011). Although *Bacteroides* species are normally commensals in the human gut flora, some of them are opportunistic pathogens that have been found in most infections of the peritoneal cavity, particularly appendicitis through abscess formation (Bennion *et al.*, 1990), and can cause anaerobic bacteremia (Merchan *et al.*, 2016).

Bacteroides possess virulence factors involved in the adherence to tissues, protection from the host's immune response and consequent destruction of tissues. The polysaccharide capsule and lipopolysachcarides (LPS) of some *Bacteroides* species protect them from the host immune response (Coyne *et al.*, 2000). LPS, also known as endotoxin, can induce a high inflammatory response when enter into the bloodstream. (Zhang *et al.*, 2009). LPS are able to affect intestinal homeostasis by either changing the permeability of or triggering immune reaction inside the intestinal mucosa. High levels of some markers of microbial translocation, including LPS, were detected in the bloodstream of subjects with CFS/ME (Giloteaux *et al.*, 2016).

Among other virulence factors, the enterotoxins and histolytic enzymes found in *Bacteroides fragilis*, namely BFT, hyaluronidase and chondroitin sulfatase, can cause tight junction destruction in gut epithelium, resulting in a "*leaky gut*" and tissue damage (Wu *et al.*, 1998; Riepe *et al.*, 1980). Moreover, it has been reported that persistent infection of mice with *Bacteroides fragilis* cause chronic colitis (Rhee *et al.*, 2009).

A proteomic analysis performed on individuals affected by Chron's disease has revealed an over-representation of many proteins, largely derived from *Bacteroides* species, which allow these opportunistic pathogens to break the intestinal barrier and invade the mucosa (Juste *et al.*, 2014).

In our study we found a significantly increased prevalence of *Bacteroides vulgatus* and unclassified *Bacteroides* in CFS patients and their relatives. These results are not totally in agreement with the findings of a previous study, which reported a reduction in *Bacteroides vulgatus* in CFS patients without IBS (Nagy-Szakal *et al.*, 2017). Our CFS population was

mainly composed by patients without IBS, nonetheless in a preliminary evaluation we did not observe any difference between CFS patients with and without IBS. An increase in *Bacteroides vulgatus* abundance was also reported in the gut of subjects with Type 1 diabetes (Higuchi *et al.*, 2018) and in children with autism spectrum disorder (Finegold *et al.*, 2010), both autoimmune conditions.

Barnesiella is a genus belonging to *Porphyromonadaceae*, that is usually found at low levels in the human gut, less than 1% of the total gut bacteria in healthy individuals, and the role of which in health is still unclear (Wylie *et al.*, 2012). *Barnesiella* produces butyric and iso-butyric acids. This genus is a poorly characterized and to date no literature data are available on its direct involvement in pathological conditions. However, *Barnesiella* may regulate the microbial composition by inhibiting the propagation of *Clostridia* and other potentially harmful microbes (Ubeda *et al.*, 2013). Accordingly, an increase in *Bacteroides*, *Barnesiella* and *Alistipes* has been reported for patients affected by *Clostridium difficile* infection (CDI), paralleled by a decrease in several bacterial groups of the phylum *Firmicutes* (Milani *et al.*, 2016).

In addition to the observed decrease in *Lachnospiraceae* and increase in *Bacteroides* and *Barnesiella* genera, in CFS patients we recorded also a large decrease in the genera *Clostridium*, *Ruminococcus* and *Dialister* and a higher prevalence of *Acidamminococcus* and *Phascolarctobacterium*. These results are similar to those reported in a study carried out on individuals with Alzheimer's disease (Vogt *et al.*, 2017). *Phascolarctobacterium* is an acetate/propionate-producer abundant in the human gut. In our study, its increase may be related to dietary habits. Indeed, a previous study demonstrated that rats fed with fat rich diets showed a higher amount of SCFAs producers, including *Phascolarctobacterium faecium* (Lecomte *et al.*, 2015). Moreover, *Phascolarctobacterium* spp. are able to use the succinate produced by other bacteria (Dot et al, 1993), such as *Bacteroides* and *Parabacteroides*, which abundances are increased by fat rich diets and are positively correlated with body weight (Lecomte *et al.*, 2015).

Another bacterium able to produce acetate and propionate is *Dialister invisus* (Downes *et al.*, 2003), which lacks in patients with Chron's disease (Joossens *et al.*, 2011).

The abundance of several *Ruminococcus* species was found to be lower in fecal samples of Chron's disease patients than in healthy control subjects (Kang *et al.*, 2010). *Ruminococcus albus* produces acetate by degrading the cellulose introduced with food (Miller *et al.*, 1995). Acetate is the most abundant SCFAs in the colon and plays a major role in the production of butyrate, which represent the first energy source for enterocytes (Louis *et al.*, 2010).

The decrease in *Bifidobacterium* that we recorded in the fecal samples of CFS/ME patients, was attributed to in the decline of *B. adolescentis*, consistently with a previous study (Giloteaux *et*

al., 2016). A significant decrease of *Bifidobacterium* was reported also in other pathological conditions, such as IBS (Tana *et al.*, 2010), IBD (Swidsinski *et al.*, 2002) and type II diabetes (Wu *et al.*, 2010). *Bifidobacteria* are a group of lactic acid-producing bacteria known to be beneficial in healthy subjects and that are widely used as probiotics [Furrie *et al.*, 2005]. Treatment with *Bifidobacterium infantis* has been reported to lower CRP levels in CFS/ME patients (Groeger *et al.*, 2013)

In the present study CFS/ME patients showed a higher abundance of *Enterobacter*, belonging to the *Proteobacteria* phylum, and in particular an increase of *Escherichia/Shigella* species. Despite these differences were not statistically significant, they are worthy of being emphasized. *Proteobacteria* includes six classes of bacteria, out of which *Gammaproteobacteria* are the most numerous and include several pathogenic bacteria such as *Escherichia coli* and *Shigella* sp. *Escherichia* and *Shigella* are more abundant in the intestinal mucosa and stool of IBD (Petersen *et al*., 2015) and Chron's disease patients (Kang *et al.*, 2010; Lapaquette *et al.*, 2010). In particular, adherent-invasive *Escherichia coli* (AIEC) has been found in IBD patients (Darfeuille-Michaud et al., 2004); AIEC can invade the intestinal epithelium, causing tissue damage and the outbreak of inflammatory processes which induce a preferential environment for other potential pathogenic bacteria.

The analysis of salivary microbiota revealed a greater species richness, than that observed in feces. The dominant phyla were, in order of abundance, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Bacteroidetes* and *TM7*. Differently from the fecal microbiota, the phyla *Acidobacteria*, *Fibrobacteres* and *Gemmatimonadales* were not detected, while *TM7* and *Fusobacteria* were found only in saliva. The comparison among CFS patients, their relatives and external controls pointed out major differences for *Actinobacteria*, which significantly increased in CFS patients.

The *Firmicutes*/*Bacteroidetes* ratio slightly increased in the oral microbiome of both CFS/ME patients and their relatives, with similar values. The increased F/B ratio observed was due to a slight decrease in *Bacteroidetes* in CFS/ME patients and in their relatives, while *Firmicutes* did not differ across the three experimental groups. In addition, *Fusobacteria* and *Proteabacteria* showed either a progressive increase or decrease, respectively. Similar changes in the relative abundances of *Fusobacteria* and *Proteobacteria* were found in a recent study on the oral microbiota of CFS/ME patients (Wang T *et al.*, 2018).

The increased abundance of *Actinobacteri*a that we observed in CFS/ME patients and, to a minor extent, in their relatives was associated with a higher prevalence of *Actinomycetales*. Within this order we observed a significant increase of *Micrococcaceae*, particularly *Rothia*

sp., only in CFS/ME patients, while *Actinomycetaceae* resulted slightly more abundant both in patients and their relatives, although the difference was not statistically significant.

Rothia is a Gram-positive and aerobic bacterial genus that normally occurs in the mouth and respiratory tract. It ferments carbohydrates and is the major producer of lactic acid. We identified two pathogenic species belonging: *Rothia dentocariosa* and *Rothia mucilaginosa*. The statistical significance was obtained only for *R. dentocariosa*.

R. dentocariosa was isolated from dental caries and is responsible of endocarditis in subjects with heart valve disorders (Ricaurte *et al.*, 2001). This species is implicated in peridontal disease, which may represent the first step in the infection of other body sites. Indeed, some findings indicate that oral bacteria are able to translocate into the bloodstream reaching various tissues, such as the brain (Singhrao *et al.*, 2015) and coronary arteries (Mougeot et al., 2017). Several studies reported that *R. dentocariosa* is involved in the infection of lung, tonsils, peritoneum and brain (Ohashi *et al.*, 2005; Morris *et al.*, 2004; Ricaurte *et al.*, 2001).

A previous study reported that *R. dentocariosa* was responsable for septic arthritis in a patient with rheumatoid arthritis (Favero *et al.*, 2009). A case of *R. dentocariosa* bacteremia has been described in a patient with ulcerative colitis (Yeung *et al.*, 2014).

Even if the increase in abundance of *Anctinomycetaceae* was not significant, it is worth noting that *Actinomyces* spp. are opportunistic pathogens that colonize the upper respiratory tract, gastrointestinal tract and female genital apparatus (Evaldson *et al.*, 1982). Some species are responsable of a chronic disease called actinomycosis, a bacterial infection of several body sites characterized by chronic granulomatous lesions and abscess formation (Vaulor *et al.*, 2014). *Actinomyces* can also cause bacteremia. *Actinomyces* species are frequently detected in the bloodstream of patients, due to increased permeability of the oral mucosa (Lockhart *et al.*, 2009).

Furthermore, in the salivary microbiota of CFS/ME patients we observed slight differences in the abundance of several other genera, such as *Haemophilus*, *Leptotrichia* and *Veillonella*. *Haemophilus*, showed the highest abundance values in external controls and the lowest in CFS/ME patients. *Veillonella* decreased only in CFS patients. The relative abundance of these two genera has been found altered in the oral microbiome of individuals positive to the human immunodeficency virus (Kistler *et al.*, 2015) and in oral lesions (Koopman *et al.*, 2015). *Leptotrichia* increased in CFS/ME patients and, to a minor extent, in their relatives compared to external controls. Species belonging to this genus are opportunistic pathogens and are associated with oral cavity abscesses and periodontal diseases (Sassone *et al.*, 2007). In addition, in immunocompromised subjects *Leptotrichia* species are involved in severe infections (septicemia) (Couturier MR, 2012).

The recorded changes in the prevalence of *Haemophilus*, *Veillonella* and *Leptotrichia* agree with the findings of a recent study carried out on Chinese CFS/ME patients (Wang *et al.*, 2018).

The fecal metabolic profile of CFS/ME patients, as assessed by UPLC-MS analysis, resulted to be different compared to that of their relatives and external controls, although the differences were not statistically significant. Interestingly, supervised PLS-DA showed that patients' relatives exhibited a metabolic profile more similar to that of external controls compared to CFS/ME patients.

Several products of bacterial metabolism contributed to the metabolic differentiation of the three groups. An overall increase of SCFAs derivatives, although no statistically significant, was observed in the CFS/ME cohort. Acetate, butyrate and propionate are the main SCFAs of the gut and it is wellknown that they are essential for its health (Wong *et al.*, 2006). Microbial SCFAs are an important energy source for intestinal cells and affect the energy metabolism of the host through their incorporation into lipids in adipocytes and oxidation in muscle cells and hepatocytes to produce glucose and glycogen (Canfora *et al.*, 2015; den Besten *et al.*, 2013). In our study we found an increase of -3-methylpentanoate (a valerate derivative) both in CFS/ME patients and in their relatives compared to controls, while no difference in isobutyrate level was observed among the three populations. In addition, CFS/ME patients showed high level of isovalerate, not observed in their relatives. Valerate is a SCFA produced in small quantities by the fermentation of carbohydrates or peptides (Bourriaud *et al.*, 2005).

The metabolites we observed in CFS/ME suggest an increase in the fermentation process that may depend on changes in the composition of the intestinal microbiota, a slowed gut transit or a high pH. Previous studies reported that the fementation process of lactate and amino acids to SCFA in the gut increases under alkaline conditions (Belenguer *et al.*, 2007).

In the present study, all amino acids levels (except for beta-N-Methylaminoalanine) slightly decreased in CFS/ME patients compared to external controls.

Beta-N-Methylaminoalanine (L-BMAA) is a non-proteinogenic amino acid produced by Cyanobacteria. It is a neurotoxin that has been postulated as a possible cause of neurodegenerative disorders, such as Alzheimer's disease, amyotrophic lateral sclerosis, and Amyotrophic Lateral Sclerosis/Parkinsonism-Dementia Complex (ALS-PDC) syndrome of Guam (Bradley et al, 2009). The observed increase in L-BMAA is an interesting outcome of this study, because many CFS/ME patients frequently report neuromotor and cognitive dysfunctions, especially during the phases of exacerbation of the disease.

Also microbial data appear consistent with an increased fermentation in CFS/ME patients, as we observed an increase of several acetate/propionate-producing species (i.e *Bacteroides*, *Phascolarctobacterium*).

Previous studies on CFS/ME patients have reported an increased production of fecal SCFAs derived from microbial fermentation, proposing a possible association with deleterious effects on the host energy metabolism (Armstrong *et al.*, 2016).

Although SCFAs have beneficial effects on human health, their increase has been observed in autism spectrum disorder (ASD) (Wang *et al.*, 2012). Indeed, the administration of propionate and SCFAs in the bloodstream of rats induce ASD with biochemical changes associated to increased oxidative stress, altered lipid profiles, innate neuroinflammatory response and mitochondrial dysfunction (Macfabe, 2012).

All considered, it is possible that the increase in intestinal permeability (Shukla *et al.*, 2015; Giloteaux *et al.*, 2016), together with the increase in SCFAs, could promote the massive transfer of SCFAs, especially acetate and propionate, into the bloodstream, causing increased oxidative stress and mitochondrial dysfunction in CFS/ME.

We found high levels of several indole derivatives only in CFS/ME patients. Indole is a metabolite mainly produced in the gut from tryptophan by microbiota activity using tryptophanase, an enzyme able to hydrolyze tryptophan to obtain indole, pyruvate, and ammonia (Lee and Lee, 2010). It has been demonstrated that indole and other metabolites derived from tryptophan can modulate inflammation in the gastrointestinal tract (Bansal *et al.*, 2010; Nicholson *et al.*, 2012) and support epithelial tight junction permeability (Keszthelyi *et al.*, 2012). Indole is absorbed at the level of the intestinal mucosa and through the entero-hepatic circulation reaches the liver where it is transformed into indican to be then excreted with urine. High levels of indican in the urine are a symptom of dysbiosis in the small intestine, and is associated to altered fermentative or putrefactive processes (Macfarlane and Macfarlane, 1997). Several bacterial species have been shown to produce large amount of indole, including both Gram-positive and Gram-negative bacteria. Among the latter, *E. coli* and *Bacteroides sp.* are able to hydrolyze tryptophan to obtain indole (Lee and Lee, 2010). The high levels of indole derivatives found in our work could be related to the increase in the abundance of the genus *E. coli*/*Shigella* and *Bacteroides sp*. observed in fecal samples from CFS/ME patients.

In subjects with CFS/ME, we also observed an increase, although not statistically significant, in biogenic amines, particularly dopamine, 3-Methoxytyramine and N-Acetylputrescine. Dopamine is the most common catecholamine in the CNS. Within the brain, dopamine acts as a neurotransmitter, through the activation of specific receptors, and plays several important roles (Vallone D *et al.*, 2000). The gut microbiota produces a wide range of mammalian

neurotransmitters, including dopamine (Clarke *et al.*, 2014). Dopamine is not able to cross the blood–brain barrier, so its synthesis and functions in peripheral areas are independent of those in the brain. A substantial amount of dopamine is released in the bloodstream performing an exocrine or paracrine function in various peripheral systems, including the immune system, reducing the activation level of lymphocytes (Sarkar *et al.*, 2010).

Consequently, changes in dopamine levels may be associated in some way to the immunological dysfunctions described in CFS/ME patients.

6. CONCLUSION

Overall, 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), our data add new information and support the autoimmune hypothesis for CFS condition (Patarca, 2001; Skowera *et al.*, 2002; Fluge *et al.*, 2011; Sotzny *et al.*, 2018) in that the intestinal microbial profile we 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 microbiota 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 sub-sample 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/ME-related immunological and cognitive dysfunctions.

It is worth emphasizing that the use of different patient inclusion/exclusion criteria and inter-study variation in sample size, experimental protocols and sequencing platforms make it

difficult to compare our results to the outcomes of previous studies. Furthermore, we also have to take into account that the presence of CFS/ME subgroups and heterogeneity in symptoms often hinder the accurate diagnosis of the disease.

The use of NGS techniques allowed us to obtain many information on the intestinal and oral microbiota composition of CFS/ME patients in a relatively short time. Few recorded differences showed statistical significance. This is likely due to both sample size and heterogeneity of the syndrome. In Italy, the lack of epidemiological studies and doctors able to recognize and diagnose the syndrome correctly makes it difficult to recruit a sufficiently large cohort of subjects.

Despite these difficulties, 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. The collaboration of the Italian Association of CFS patients - AMCFS Onlus, which relies on a team of experienced doctors and is committed to the development of CFS/ME guidelines, was very helpful for getting in touch with patient and their relatives.

In conclusion, 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.

7. REFERENCES

Aagaard K, Ma J, Antony KM, et al. The placenta harbors a unique microbiome. *Sci Transl Med*., 2014; 6(237):237ra65. doi:10.1126/scitranslmed.3008599.

Aaron LA, Burke MM, Buchwald D. Overlapping conditions among patients with chronic fatigue syndrome, fibromyalgia, and temporomandibular disorder. *Arch Intern Med*., 2000; 160(2):221-227. doi:10.1001/archinte.160.2.221.

Abe K, Takahashi A, Masashi Fujita M. Dysbiosis of oral microbiota and its association with salivary immunological biomarkers in autoimmune liver disease. *PLoS One*.,2018;13(7): e0198757.doi:10.1371/journal.pone.0198757.

Ablashi DV, Eastman HB, Owen CB, et al. Frequent HHV-6 reactivation in multiple sclerosis (MS) and chronic fatigue syndrome (CFS) patients. *J. Clin. Virol.*, 2000; 16(3):179–191.doi:10.1016/s1386-6532(99)00079-7.

Albrecht RM, Oliver VL, Poskanzer DC. Epidemic neuromyasthenia. Outbreak in a convent in New York state. *JAMA*., 1964; 187(12): 904–907. doi:10.1001/jama.1964.03060250022005.

Albright F, Light K, Light A, et al. Evidence for a heritable predisposition to chronic fatigue syndrome. *BMC Neurol.*, 2011; 11:62. doi: 10.1186/1471-2377-11-62.

Armstrong CW, McGregor NR, Lewis DP, et al. The association of fecal microbiota and fecal, blood serum and urine metabolites in myalgic encephalomyelitis/chronic fatigue syndrome. *Metabolomics.*, 2017; 13:8. doi: 10.1007/s11306-016-1145-z.

Arumugam M, Raes J, Pelletier E. Enterotypes of the human gut microbiome. *Nature*, 2011; 47388(7346):174-180. doi: 10.1038/nature09944.

Bakker GJ, Nieuwdorp M. Fecal microbiota transplantation: therapeutic potential for a multitude of diseases beyond *Clostridium difficile*. *Microbiol Spectr.*, 2017; 5(4). doi: 10.1128/microbiolspec.BAD-0008-2017.

Bansal T, Alaniz RC, Wood TK, et al. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. *Proc Natl Acad Sci U S A.*, 2010; 107:228–233. doi: 10.1073/pnas.0906112107.

Bao GJ, Kari K, Tervahartiala T, et al. Meurman. Proteolytic activities of oral bacteria on prommp-9 and the effect of synthetic proteinase inhibitors. *The Open Dentistry Journal*, 2008; 2:96-102. doi: 10.2174/1874210600802010096.

Bassis C, Young V, Schmidt T. Methods for characterizing microbial communities associated with the human body. *The Human Microbiota.*, 2013; 51–74. doi: 10.1002/9781118409855.ch2.

Belenguer A, Duncan SH, Holtrop G, et al. Impact of pH on lactate formation and utilization by human fecal microbial communities. *Appl Environ Microbiol.*, 2007; 73(20):6526-33. doi: 10.1128/AEM.00508-07.

Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell*., 2014; 157(1):121–41. doi:10.1016/j.cell.2014.03.011.

Benítez-Páez A, Gómez Del Pulgar EM, Sanz Y. The Glycolytic Versatility of Bacteroides uniformis CECT 7771 and Its Genome Response to Oligo and Polysaccharides. *Front Cell Infect Microbiol*., 2017; 7:383. doi:10.3389/fcimb.2017.00383

Bennion RS, Baron EJ, Thompson JE Jr, et al. The bacteriology of gangrenous and perforated appendicitis--revisited. *Ann Surg.*, 1990; 211(2):165-71. doi:10.1097/00000658-199002000-00008.

Bergmann H, Rodriguez JM, Salminen S, et al. Probiotics in human milk and probiotic supplementation in infant nutrition: a workshop report. *Br J Nutr*., 2014; 112(7):1119–1128.doi:10.1017/S0007114514001949.

Berry D, Mahfoudh K B, Wagner M, et al. Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. Appl. *Environ. Microbiol.*, 2011; 77, 7846–7849.doi: 10.1128/AEM.05220-11.

Bingol K, Bruschweiler R. Knowns and unknowns in metabolomics identified by multidimensional NMR and hybrid MS/NMR methods. *Curr. Opin. Biotechnol.*, 2017; 43:17. doi: 10.1016/j.copbio.2016.07.006.

Blundell S, Ray KK, Buckland M, et al. Chronic fatigue syndrome and circulating cytokines: A systematic review. *Brain behavior and immunity*, 2015; 50:186-195. doi:10.1016/j.bbi.2015.07.004.

Boccard J, Veuthey JL, Rudaz S. Knowledge discovery in metabolomics: An overview of MS data handling. *J. Sep. Sci.*, 2010; 33:290–304. doi: 10.1002/jssc.200900609.

Borody TJ, Nowak A, Finlayson S. The GI microbiome and its role in chronic fatigue syndrome: a summary of bacteriotherapy. *ACNEM J.*, 2012; 31(3):3–8.

Bourriaud C, Robins RJ, Martin L, et al. Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident. *J Appl Microbiol.*, 2005; 99(1):201-12. doi:10.1111/j.1365-2672.2005.02605.x.

Bradley WG, Mash, DC. Beyond Guam: the cyanobacterial/BMAA hypothesis of the cause of ALS and other neurodegenerative diseases. *ALS.*, 2009; 10:7–20. doi:10.3109/17482960903286009.

Buchwald D, Herrell R, Ashton S, et al. A twin study of chronic fatigue. *Psychosom Med*., 2001; 63(6):936-943. doi:10.1097/00006842-200111000-00012.

Byers HL, Tarelli E, Homer KA, et al. Sequential deglycosylation and utilization of the n-linked, complex-type glycans of human alpha1-acid glycoprotein mediates growth of Streptococcus oralis. *Glycobiology*, 1999; 9(5):469-479. doi:10.1093/glycob/9.5.469.

Canfora EE, Jocken JW, Blaak EE. Short-chain fatty acids in control of body weight and insulin sensitivity. *Nat Rev Endocrinol.*, 2015; 11(10):577-91. doi: 10.1038/nrendo.2015.128.

Cano GP, Santacruz A, Moya Á, et al. Bacteroides uniformis CECT 7771 ameliorates metabolic and immunological dysfunction in mice with high-fat-diet induced obesity. *PLoS One.*, 2012; 7(7): e41079. doi:10.1371/journal.pone.0041079.

Capelli E, Lorusso L, Ghitti M, et al. Chronic fatigue syndrome: Features of a population of patients from northern Italy. *International Journal of Immunopathology and Pharmacology*, 2015; 28(1):53 –59. doi: 10.1177/0394632015572074.

Capelli E, Zola R, Lorusso L, et al. Chronic fatigue syndrome/myalgic encephalomyelitis: an update. *Int J Immunopathol Pharmacol.*, 2010; 23(4):981-989. doi:10.1177/039463201002300402.

Carlo-Stella N, Badulli C, De Silvestri A, et al. A first study of cytokine genomic polymorphisms in CFS: positive association of TNF-857 and IFN γ874 rare alleles. *Clin Exp Rheumathol*., 2006; 24(2):179-182.

Carruthers BM, van de Sande MI, de Meirleir KL, et al. Myalgic encephalomyelitis: International consensus criteria. *J. Intern. Med*., 2011; 270(4):327–338. doi: 10.1111/j.1365-2796.2011.02428.x.

Cavestro GM, Ferrarese R, Petrone M, et al. Variations of Oral and Fecal Microbiota Are Associated With Autoimmune Pancreatitis. *Gastroenterology*., 2016; 150(4):S512-S3. doi: https://doi.org/10.1016/S0016-5085(16)31762-0

Chalder T, Berelowitz G, Pawlikowska T, et al. Development of a fatigue scale. *J Psychosom Res.*, 1993; 37(2):147-53. DOI: 10.1016/0022-3999(93)90081-p

Chen J, Talwalkar JA, Yin M, et al. Early detection of nonalcoholic steatohepatitis in patients with nonalcoholic fatty liver disease by using MR elastography. *Radiology.*, 2011; 259(3):749-56. doi:10.1148/radiol.11101942.

Chen R, Liang FX, Moriya J, et al. Chronic fatigue syndrome and the central nervous system. *J Int Med Res.*, 2008; 36:867-74. doi: 10.1177/147323000803600501.

Chia JK. The role of enterovirus in chronic fatigue syndrome. *J. Clin. Pathol.*, 2005; 58(11):1126–1132. doi: 10.1136/jcp.2004.020255.

Clarke G, Stilling RM, Kennedy PJ, et al. Minireview: Gut Microbiota: The Neglected Endocrine Organ. *Mol Endocrinol*., 2014; 28(8):1221-1238. doi: 10.1210/me.2014-1108.

Clauw DJ, Chrousos GP. Chronic pain and fatigue syndromes: overlapping clinical and neuroendocrine features and potential pathogenic mechanisms. *Neuroimmunomodulation*, 1997; 4(3):134-153. doi:10.1159/000097332.

Cleare AJ. The neuroendocrinology of chronic fatigue syndrome. *Endocr Rev.*, 2003; 24(2):236-52. doi:10.1210/er.2002-0014.

Clemente JC, Ursell LK, Parfrey LW, et al. The impact of the gut microbiota on human health: an integrative view. *Cell.*, 2012; 148(6):1258–70. doi:10.1016/j.cell.2012.01.035.

Clish CB. Metabolomics: an emerging but powerful tool for precision medicine. *Cold Spring Harb Mol Case Stud*., 2015; 1(1): a000588. doi: 10.1101/mcs.a000588.

Couturier MR, Slechta ES, Goulston C, et al. Leptotrichia bacteremia in patients receiving high-dose chemotherapy. *J Clin Microbiol.*, 2012; 50(4):1228-32. doi: 10.1128/JCM.05926-11.

Coyne MJ, Tzianabos AO, Mallory BC, et al. Polysaccharide biosynthesis locus required for virulence of Bacteroides fragilis. *Infect Immun.*, 2001; 69(7):4342-50. doi:10.1128/IAI.69.7.4342-4350.2001.

Cryan JF1, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci*., 2012;13(10):701-12. doi: 10.1038/nrn3346.

Darfeuille-Michaud A, Boudeau J, Bulois P, et al. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology.*, 2004; 127(2):412-21. doi:10.1053/j.gastro.2004.04.061.

Darveau RP. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol.*, 2010; 8(7):481-90. doi: 10.1038/nrmicro2337.

De Filippo C, Cavalieri D, Di Paola M. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences*, 2010; 107(33):14691-14696. doi: 10.1073/pnas.1005963107.

De Groot PF, Frissen MN, De Clercq NC, et al. Fecal microbiota transplantation in metabolic syndrome: history, present and future. *Gut Microbes.*, 2017; 8(3):253–267. doi: 10.1080/19490976.2017.1293224.

Demitrack MA, Dale JK, Straus SE, et al. Evidence for impaired activation of the hypothalamic-pituitary-adrenal axis in patients with chronic fatigue syndrome. *J Clin Endocrinol Metab.*, 1991; 73(6):1224-34. doi:10.1210/jcem-73-6-1224.

Den Besten G, Van Eunen K, Groen AK, et al. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res.*, 2013; 54(9): 2325-40. doi:10.1194/jlr.R036012.

DeSantis TZ, Hugenholtz P, Keller K, et al. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Res*., 2006; 34: W394–W399. doi: 10.1093/nar/gkl244.

Diaz Heijtz R, Wang S, Anuar F, et al. Normal gut microbiota modulates brain development and behavior. *Proc. Natl. Acad. Sci. USA*, 2011; 108(7):3047–3052. doi: 10.1073/pnas.1010529108.

Diaz PI, Chalmers NI, Rickard AH, et al. Molecular characterization of subject-specific oral microflora during initial colonization of enamel. *Applied and Environmental Microbiology*, 2006; 72(4):2837-2848. doi:10.1128/AEM.72.4.2837-2848.2006.

Docktor MJ, Paster BJ, Abramowicz S, et al. Alterations in diversity of the oral microbiome in pediatric inflammatory bowel disease. *Inflammatory bowel diseases*., 2012; 18(5):935-42. doi: 10.1002/ibd.21874.

Dominguez-Bello MG, Costello EK, Contreras M. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci USA*, 2010; 107(26):11971-11975. doi: 10.1073/pnas.1002601107.

Dot TD, Osawa R, Stackebrandt E. Phascolarctobacterium faecium gen. nov, spec. nov., a Novel Taxon of the Sporomusa Group of Bacteria. *Syst Appl Microbiol.*, 1993; 16:380–384. doi: 10.1016/S0723-2020(11)80269-9.

Downes J, Munson M, Wade WG. Dialister invisus sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol.*, 2003; 53(Pt 6):1937- 40. doi: 10.1099/ijs.0.02640-0.

Duncan SH, Hold GL, Barcenilla A, et al. Roseburia intestinalis sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. *Int J Syst Evol Microbiol*., 2002; 52(Pt 5):1615-20. doi:10.1099/00207713-52-5-1615.

Duncan SH, Lobley GE, Holtrop G, et al. Human colonic microbiota associated with diet, obesity and weight loss. *Int J Obes (Lond).*, 2008; 32(11):1720-4. doi: 10.1038/ijo.2008.155.

Edgar RC, Haas B J, Clemente J C, et al. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics, 2011; 27:2194–2200. doi: 10.1093/bioinformatics/btr381.

Evaldson G, Heimdahl A, Kager L, et al. The normal human anaerobic microflora. *Scand J Infect Dis Suppl.*, 1982; 35:9-15.

Favero M, Raffeiner B, Cecchin D, et al. Septic Arthritis Caused by Rothia dentocariosa in a Patient with Rheumatoid Arthritis Receiving Etanercept Therapy. *The Journal of Rheumatology.*, 2009; 36(12):2846–2847. doi:10.3899/jrheum.090276.

Finegold SM, Dowd SE, Gontcharova V, et al. Pyrosequencing study of fecal microflora of autistic and control children. *Anaerobe*, 2010; 16(4):444-53. doi: 10.1016/j.anaerobe.2010.06.008.

Fletcher MA, Zeng XR, Maher K, et al. Biomarkers in chronic fatigue syndrome: Evaluation of natural killer cell function and dipeptyl peptidase IV. *PLoS ONE*, 2010; 5: e10817.doi:10.1371/journal.pone.0010817.

Fluge Ø; Bruland O; Risa K; et al. Benefit from B-lymphocyte depletion using the anti-CD20 antibody rituximab in chronic fatigue syndrome. A double-blind and placebo-controlled study. *PLoS ONE*, 2011; 6(10):e26358. doi: 10.1371/journal.pone.0026358.

Fond G, Boukouaci W, Chevalier G, et al. The "psychomicrobiotic": Targeting microbiota in major psychiatric disorders: A systematic review. *Pathol. Biol. (Paris)*, 2015; 63:35–42. doi: 10.1016/j.patbio.2014.10.003.

Francino MP. Early development of the gut microbiota and immune health. *Pathogens,* 2014; 38(3):769–90. doi:10.3390/pathogens3030769.

Frank DN, Amand AL, Feldman RA, et al. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A.*, 2007; 104(34):13780-5. doi:10.1073/pnas.0706625104.

Fremont M, Coomans D, Massart S, et al. High-throughput 16s rRNA gene sequencing reveals alterations of intestinal microbiota in myalgic encephalomyelitis/chronic fatigue syndrome patients. *Anaerobe*, 2013; 22:50–56. doi:10.1016/j.anaerobe.2013.06.002.

Fremont M, Metzger K, Rady H, et al. Detection of herpesviruses and parvovirus B19 in gastric and intestinal mucosa of chronic fatigue syndrome patients. *In Vivo*, 2009; 23(2):209–213.

Frissora CL, Koch KL. Symptom overlap and comorbidity of irritable bowel syndrome with other conditions. Curr. *Gastroenterol. Rep*., 2005; 7(4):264-271. doi:10.1007/s11894-005-0018-9.

Fukuda K, Straus SE, Hickie I, et al. The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group. *Ann Intern Med*., 1994; 121(12):953–9. doi:10.7326/0003-4819-121-12-199412150-00009.

Furrie E, Macfarlane S, Kennedy A, et al. Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut.*, 2005; 54(2):242–9. doi: 10.1136/gut.2004.044834.

Gebregiworgis T, Powers R. Application of NMR metabolomics to search for human disease biomarkers. Comb. *Chem. High Throughput Screening*, 2012; 15(8):595–610. doi:10.2174/138620712802650522.

Giloteaux L, Goodrich JK, Walters WA, et al. Reduced diversity and altered composition of the gut microbiome in individuals with myalgic encephalomyelitis/chronic fatigue syndrome. *Microbiome*, 2016; 4(1):30.

Glaser R, Litsky ML, Padgett DA, et al. EBV-encoded dUTPase induces immune dysregulation: Implications for the pathophysiology of EBV-associated disease. *Virology*, 2006; 346(1):205–218.doi:10.1016/j.virol.2005.10.034.

Glaser R, Padgett DA, Litsky ML, et al. Stress-associated changes in the steady-state expression of latent epstein-barr virus: Implications for chronic fatigue syndrome and cancer. *Brain Behav. Immun.*, 2005; 19(2):91–103. doi:10.1016/j.bbi.2004.09.001.

Gordon JI, Dewey KG, Mills DA, et al. The human gut microbiota and undernutrition. *Sci Transl Med*., 2012; 4(137):137. doi:10.1126/scitranslmed.3004347.

Gorvitovskaia A, Holmes SP, Huse SM. Interpreting Prevotella and Bacteroides as biomarkers of diet and lifestyle. *Microbiome.*, 2016; 4:1-15. doi: 10.1186/s40168-016-0160-7.

Gosalbes MJ1, Durbán A, Pignatelli M, et al. Metatranscriptomic approach to analyze the functional human gut microbiota. *PLoS One*., 2011; 6(3):e17447. doi:10.1371/journal.pone.0017447.

Groeger D, O'Mahony L, Murphy EF, et al. Bifidobacterium infantis 35624 modulates host inflammatory processes beyond the gut. *Gut Microbes.*, 2013; 4(4):325–39. doi:10.4161/gmic.25487.

Gunn WJ, Connell DB, Randall B. Epidemiology of chronic fatigue syndrome: the centers for disease control study. *Ciba Found Symp.*, 1993; 173:83-101. doi:10.1002/9780470514382.ch6.

Hevia A, Milani C, López P, et al. Intestinal dysbiosis associated with systemic lupus erythematosus. *MBio.*, 2014; 5(5):e01548-14. doi: 10.1128/mBio.01548-14.

Hickie I, Davenport T, Wakefield D, et al. Post-infective and chronic fatigue syndromes precipitated by viral and non-viral pathogens: Prospective cohort study. *BMJ,* 2006; 333(7568):575.doi:10.1136/bmj.38933.585764.AE.

Hietbrink F, Besselink MG, Renooij W, et al. Systemic inflammation increases intestinal permeability during experimental human endotoxemia. *Shock,* 2009; 32(4):374–378.doi: 10.1097/SHK.0b013e3181a2bcd6.

Holmes GP, Kaplan JE, Gantz NM, et al. Chronic fatigue syndrome: a working case definition. *Ann Intern Med.,* 1988; 108(3):387-89. doi: 10.7326/0003-4819-108-3-387.

Homer KA, Whiley RA, Beighton D. Proteolytic activity of oral streptococci. *FEMS Microbiology Letters,* 1990; 55(3):257-260. doi:10.1016/0378-1097(90)90005-b.

Hornig M, Montoya JG, Klimas NG, et al. Distinct plasma immune signatures in ME/CFS are present early in the course of illness. *Sci Adv.,* 2015; 1(1). doi: 10.1126/sciadv.1400121.

Hugenholtz P, Goebel BM, Pace NR. Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity. *Journal of Bacteriology.,* 1998; 180(18):4765–4774.

Ianiro G, Tilg H, Gasbarrini A. Antibiotics as deep modulators of gut microbiota: between good and evil. *Gut.,* 2016; 65(11):1906-1915. doi: 10.1136/gutjnl-2016-312297.

IoM: Beyond myalgic encephalomyelitis/chronic fatigue syndrome: Redefining an illness. Washington, D.C.: *The National Academies Press.,* 2015.

Ismail AN, Ragab SH, Abd Elbaky A, et al. Frequency of Firmicutes and Bacteroidetes in gut microbiota in obese and normal weight Egyptian children and adults. *Arch Med Sci.,* 2011; 7(3):501-7. doi:10.5114/aoms.2011.23418.

Jandhyala SM, Talukdar R, Subramanyam C, et al. Role of the normal gut microbiota. *World J Gastroenterol.,* 2015; 21(29):8787-8803. doi: 10.3748/wjg.v21.i29.8787.

Jason L, Torres-Harding S, Njok M. The face of CFS in the U.S. *CFIDS Chronicle,* 2006; pp. 16–21.

Jason LA, Benton MC, Valentine L, et al. The economic impact of ME/CFS: Individual and societal costs. *Dyn Med.,* 2008; 7:6. doi: 10.1186/1476-5918-7-6.

Jones DEJ, Hollingsworth KG, Taylor R, et al. Abnormalities in Ph handling by peripheral muscle and potential regulation by the autonomic nervous system in chronic fatigue syndrome. *J Int Med.,* 2009; 267:394–401. doi: 10.1111/j.1365-2796.2009.02160.x.

Joossens M, Huys G, Cnockaert M, et al. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut.*, 2011 May; 60(5):631-7. doi: 10.1136/gut.2010.223263.

Juste C, Kreil DP, et al. Bacterial protein signals are associated with Crohn's disease. *Gut.*,2014; 63(10). doi: 10.1136/gutjnl-2012-303786.

Kamada N, Chen GY, Inohara N, et al. Control of Pathogens and Pathobionts by the Gut Microbiota. *Nat Immunol*., 2013; 14(7): 685-690. doi: 10.1038/ni.2608.

Kang S, Denman SE, Morrison M, et al. Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom phylogenetic microarray. *Inflamm Bowel Dis.*, 2010; 16(12):2034-42. doi: 10.1002/ibd.21319.

Karl JP, Hatch AM, Arcidiacono SM, et al. Effects of Psychological, Environmental and Physical Stressors on the Gut Microbiota. *Front Microbiol*., 2018; 9:2013. doi: 10.3389/fmicb.2018.02013.

Kau AL, Ahern PP, Griffin NW, et al. I. Human nutrition, the gut microbiome and the immune system. *Nature*, 2011; 474(7351):327–336. doi: 10.1038/nature10213.

Kaur R, Gilbert SC, Sheehy EC, et al. Salivary levels of Bifidobacteria in caries-free and cariesactive children. *International journal of paediatric dentistry*., 2013; 23(1):32-8. doi: 10.1111/j.1365-263X.2011.01220.x.

Kelly CR, Kahn S, Kashyap P, et al. Update on Fecal Microbiota Transplantation 2015: Indications, Methodologies, Mechanisms, and Outlook. *Gastroenterology*, 2015; 149(1):223-37. doi: 10.1053/j.gastro.2015.05.008.

Keszthelyi D, Troost FJ, Jonkers DM, et al. Does acute tryptophan depletion affect peripheral serotonin metabolism in the intestine? *Am J Clin Nutr*., 2012; 95:603–608. doi:10.3945/ajcn.111.028589.

Kistler JO, Pesaro M, Wade WG. Development and pyrosequencing analysis of an in-vitro oral biofilm model. *BMC Microbiol.*, 2015; 15:24. doi: 10.1186/s12866-015-0364-1.

Koliada A, Syzenko G, Moseiko V, et al. Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. *BMC Microbiol*., 2017; 17(1):120. doi:10.1186/s12866-017-1027-1.

Koopman JE, van der Kaaij NC, Buijs MJ, et al. The Effect of Fixed Orthodontic Appliances and Fluoride Mouthwash on the Oral Microbiome of Adolescents - A Randomized Controlled Clinical Trial. *PLoS One.*, 2015; 10(9):e0137318. doi: 10.1371/journal.pone.0137318.

Kuehbacher T, Rehman A, Lepage P, et al. Intestinal TM7 bacterial phylogenies in active inflammatory bowel disease. *J Med Microbiol*., 2008; 57:1569–1576. doi: 10.1099/jmm.0.47719-0.

Labus JS, Hollister EB, Jacobs J, et al. Differences in gut microbial composition correlate with regional brain volumes in irritable bowel syndrome. *Microbiome,* 2017; 5(1):49. doi: 10.1186/s40168-017-0260-z.

Lakhan SE, Kirchgessner A. Gut inflammation in chronic fatigue syndrome. *Nutr Metab.,* 2010; 7:79. doi: 10.1186/1743-7075-7-79.

Lapaquette P, Glasser AL, Huett A, et al. Crohn's disease-associated adherent-invasive E. coli are selectively favoured by impaired autophagy to replicate intracellularly. *Cell Microbiol*., 2010; 12(1):99-113. doi: 10.1111/j.1462-5822.2009.01381.x.

Larsen N, Vogensen FK, Van den Berg FW, et al. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PLoS One*., 2010; 5(2):e9085. doi:10.1371/journal.pone.0009085.

Lazar V, Ditu LM, Pircalabioru GG, et al. Aspects of gut microbiota and immune system interactions in infectious diseases, immunopathology, and cancer. *Front Immunol*., 2018; 9:1830. doi: 10.3389/fimmu.2018.01830.

Lecomte V, Kaakoush NO, Maloney CA, et al. Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. *PLoS One*., 2015; 10: e126931. doi:10.1371/journal.pone.0126931.

Lee JH, Lee J. Indole as an intercellular signal in microbial communities. *FEMS Microbiol Rev*., 2010; 34:426–444. doi: 10.1111/j.1574-6976.2009.00204.x.

Leshem A, Horesh N, Elinav E. Fecal Microbial Transplantation and Its Potential Application in Cardiometabolic Syndrome. *Front Immunol.,* 2019; 10:1341. doi: 10.3389/fimmu.2019.01341.

Liu Z, DeSantis TZ, Andersen GL. Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers. *Nucleic Acids Res.,* 2008; 36(18): e120. doi: 10.1093/nar/gkn491.

Lockhart PB, Brennan MT, Thornhill M, et al. Poor oral hygiene as a risk factor for infective endocarditis-related bacteremia. *J Am Dent Assoc,* 2009; 140(10):1238-44. doi:10.14219/jada.archive.2009.0046.

Lora V. Hooper, Tore Midtvedt, Jeffrey I. GordonHow host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu. Rev. Nutr*., 2002; 22:283–307 doi: 10.1146/annurev.nutr.22.011602.092259.

Lorusso L, Mikhaylova SW, Capelli E, et al. Immunological aspects of chronic fatigue syndrome. *Autoimmun Rev.*, 2009; 8:287-91. doi: 10.1016/j.autrev.2008.08.003.

Lorusso L, Rusticali B, Tavini G, et al. Fattori epidemiologici in Italia. In: Chronic Fatigue Syndrome "CFS". Rome: Agenzia Nazionale per i Servizi Sanitari Regionali, 2014; pp. 53–58. Available at: www.Agenas.it/Chronic Fatigue Syndrome.

Louis P, Young P, Holtrop G, et al. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. *Environ Microbiol.*, 2010; 12(2):304-14. doi: 10.1111/j.1462-2920.2009.02066.x.

Macfabe D F. Short-chain fatty acid fermentation products of the gut microbiome: implications in autism spectrum disorders. *Microbial Ecology in Health and Disease,*2012; 23:19260. doi:10.3402/mehd.v23i0.19260.

Macfarlane GT, Macfarlane S. Human colonic microbiota: ecology, physiology and metabolic potential of intestinal bacteria. *Scand J Gastroenterol Suppl.*, 1997; 222:3-9. doi: 10.1080/00365521.1997.11720708.

Machiels K, Joossens M, Sabino J, et al. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. *Gut.*, 2014; 63(8):1275-83. doi: 10.1136/gutjnl-2013-304833.

Mandal M, Olson DJ, Sharma T, et al. Butyric acid induces apoptosis by up-regulating Bax expression via stimulation of the c-Jun N-terminal kinase/activation protein-1 pathway in human colon cancer cells. *Gastroenterology,* 2001; 120(1):71-8. doi: 10.1053/gast.2001.20897.

Manichanh C, Rigottier-Gois L, Bonnaud E, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut.*, 2006; 55(2):205–211.doi:10.1136/gut.2005.073817.

Marasco G, Di Biase AR, Schiumerini R, et al. Gut Microbiota and Celiac Disease. *Dig Dis Sci.*, 2016; 61(6):1461-72. doi: 10.1007/s10620-015-4020-2.

Masella AP, Bartram AK, Truszkowski JM, et al. PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics*, 2012; 13:31. doi: 10.1186/1471-2105-13-31.

Maukonen J, Kolho KL, Paasela M, et al. Altered Fecal Microbiota in Paediatric Inflammatory Bowel Disease. *J Crohns Colitis.*, 2015; 9(12):1088-95. doi: 10.1093/ecco-jcc/jjv147.

McDonald D, Price MN, Goodrich J, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME Journal*, 2011; 6:610–618. doi: 10.1038/ismej.2011.139.

Meehan CJ, Beiko RG. A Phylogenomic View of Ecological Specialization in the Lachnospiraceae, a Family of Digestive Tract-Associated Bacteria. *Genome Biol Evol.*, 2014; 6(3):703–713. doi: 10.1093/gbe/evu050.

Merchan C, Parajuli S, Siegfried J, et al. Multidrug-Resistant Bacteroides fragilis Bacteremia in a US Resident: An Emerging Challenge. *Case Rep Infect Dis*., 2016; 2016:3607125. doi:10.1155/2016/3607125.

Milani C, Ticinesi A, Gerritsen J, et al. Gut microbiota composition and Clostridium difficile infection in hospitalized elderly individuals: a metagenomic study. *Sci Rep.*, 2016; 6:25945. doi: 10.1038/srep25945.

Miller TL, Currenti E, Wolin MJ. Anaerobic bioconversion of cellulose by Ruminococcus albus, Methanobrevibacter smithii, and Methanosarcina barkeri. *Appl Microbiol Biotechnol.*, 2000; 54(4):494-8. doi: 10.1007/s002530000430.

Morgan XC, Tickle TL, Sokol H, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol.*, 2012; 13(9): R79. doi: 10.1186/gb-2012-13-9-r79.

Morris SK, Nag S, Suh KN, et al. Recurrent chronic ambulatory peritoneal dialysis-associated infection due to rothia dentocariosa. *Can J Infect Dis Med Microbiol*., 2004; 15(3):171–3.doi:10.1155/2004/823463.

Mougeot LC, Stevens CB, Paster BJ, et al. Porphyromonas gingivalis is the most abundant species detected in coronary and femoral arteries. *J. Oral Microbiol.,*2017; 9:1281562. doi:10.1080/20002297.2017.1281562.

Moye ZD, Zeng L, Burne RA. Fueling the caries process: carbohydrate metabolism and gene regulation by Streptococcus mutans. *Journal of oral microbiology*, 2014; 6.doi: 10.3402/jom.v6.24878.

Myhill S, Booth NE, McLaren-Howard J. Chronic fatigue syndrome and mitochondrial dysfunction. *Int J Clin Exp Med.*, 2009; 2(1):1-16.

Nacul LC, Lacerda EM, Pheby D, et al. Prevalence of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) in three regions of England: a repeated cross-sectional study in primary care. *BMC Med.*, 2011; 9:91. doi: 10.1186/1741-7015-9-91.

Nagy K, Sonkodi I, Szoke I, et al. The microflora associated with human oral carcinomas. *Oral Oncol*., 1998; 34(4):304–8. doi:10.1016/S1368-8375(98)80012-2.

Nagy-Szakal D, Williams BL, Mishra N, et al. Fecal metagenomic profiles in subgroups of patients with myalgic encephalomyelitis/ chronic fatigue syndrome. *Microbiome*, 2017; 5(1):44. doi: 10.1186/s40168-017-0261-y.

Nasidze I, Li J, Quinque D, et al. Global diversity in the human salivary microbiome. *Genome Research*, 2009; 19(4):0636-643. doi: 10.1101/gr.084616.108.

Ng J, Ng LK, Mayrand D, et al. Aminopeptidase activities in Peptostreptococcus spp. Are statistically correlated to gelatin hydrolysis. *Canadian Journal of Microbiology*, 1998; 44(3):303-306.

Nicholson, JK, Holmes E, Kinross J, et al. Host-gut microbiota metabolic interactions. *Science*, 2012; 336(6086):1262–1267. doi:10.1126/science.1223813.

Nijs J, Nees A, Paul L, et al. Altered immune response to exercise in patients with chronic fatigue syndrome/myalgic encephalomyelitis: A systematic literature review. *Exerc Immunol Rev.*, 2014; 20:94–116.

Ohashi M, Yoshikawa T, Akimoto S, et al. Severe acute tonsillitis caused by Rothia dentocariosa in a healthy child. *The Pediatric Infectious Disease Journal.*, 2005; 24(5): 466–467. doi:10.1097/01.inf.0000160958.26544.38.

Patarca R. Cytokines and chronic fatigue syndrome. *Ann N Y Acad Sci.*, 2001; 933:185-200. doi:10.1111/j.1749-6632.2001.tb05824.x.

Paulson JN, Pop M, Bravo HC. Metastats: an improved statistical method for analysis of metagenomic data. *Genome Biol*., 2011; 12:1–27. doi: https://doi.org/10.1186/gb-2011-12-s1-p17.

Petersen AM, Halkjær SI, Gluud LL. Intestinal colonization with phylogenetic group B2 Escherichia coli related to inflammatory bowel disease: a systematic review and meta-analysis. *Scand J Gastroenterol.*, 2015; 50(10):1199-207. doi: 10.3109/00365521.2015.1028993.

Pruesse E, Quast C, Knittel K, et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.*, 2007; 35:7188–7196. doi: 10.1093/nar/gkm864.

Qin J, Li R, Raes J, et al. A human gut microbial gene catalog established by metagenomic sequencing. Nature, 2010; 464(7285):59-65. doi: 10.1038/nature08821.

Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*, 2012; 490(7418):55–60. doi: 10.1038/nature11450.

Raghavendran K, Mylotte JM, Scannapieco FA. Nursing home-associated pneumonia, hospitalacquired pneumonia and ventilator-associated pneumonia: the contribution of dental biofilms and periodontal inflammation. *Periodontol.*, 2007; 2000(44):164–177. doi: 10.1111/j.1600-0757.2006.00206.x.

Rao AV, Bested AC, Beaulne TM, et al. A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. *Gut Pathog.*, 2009; 1(1):6. doi: 10.1186/1757-4749-1-6.

Rautava J, Pinnell LJ, Vong L, et al. Oral microbiome composition changes in mouse models of colitis. *Journal of gastroenterology and hepatology*, 2015; 30(3):521-7. doi: 10.1111/jgh.12713.

Reyes M, Gary JHE, Dobbins JG, et al. Surveillance for chronic fatigue syndrome: four U.S. cities. *MMWR CDC Surveill Summ*., 1997; 46(2):1-13.

Reyes M, Nisenbaum R, Hoaglin D, et al. Prevalence and incidence of chronic fatigue syndrome in Wichita, Kansas. *Arch. Intern. Med.*, 2003; 163(13):1530-6. doi:10.1001/archinte.163.13.1530.

Rhee KJ, Wu S, Wu X, et al. Induction of persistent colitis by a human commensal, enterotoxigenic Bacteroides fragilis, in wild-type C57BL/6 mice. *Infect Immun.*, 2009; 77(4):1708-18. doi:10.1128/IAI.00814-08.

Ricaurte JC, Klein O, LaBombardi V, et al. Rothia dentocariosa endocarditis complicated by multiple intracranial hemorrhages. *South Med J.*, 2001; 94(4):438-40.

Ridaura VK, Faith JJ, Rey FE, et al. Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science*, 2013; 341(6150):1241214. doi: 10.1126/science.1241214.

Riedl A, Schmidtmann M, Stengel A, et al. Somatic comorbidities of irritable bowel syndrome: a systematic analysis. *J Psychosom Res*., 2008; 64(6):573–82. doi:10.1016/j.jpsychores.2008.02.021.

Riekeberg E, Powers R. New frontiers in metabolomics: from measurement to insight. *F1000Research*., 2017; 6:1148. doi: 10.12688/f1000research.11495.1.

Riepe SP, Goldstein J, Alpers DH. Effect of secreted Bacteroides proteases on human intestinal brush border hydrolases. *J. Clin. Investig.*, 1980; 66:314-322. doi:10.1172/JCI109859.

Rinninella E, Raoul P, Cintoni M, et al. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms*., 2019;7(1). doi: 10.3390/microorganisms7010014

Robinson CJ, Bohannan BJM, Young VB. From structure to function: the ecology of host-associated microbial communities. *Microbiol. Mol. Biol. Rev*., 2010; 74(3):453–476.doi: 10.1128/MMBR.00014-10.

Rogers AH, Gunadi A, Gully NJ, et al. An aminopeptidase nutritionally important to Fusobacterium nucleatum. *Microbiology*, 1998; 144(Pt7):1807-1813. doi:10.1099/00221287-144-7-1807.

Rowland I, Gibson G, Heinken A, et al. Gut microbiota functions: metabolism of nutrients and other food components. *Eur J Nutr*., 2018; 57(1):1-24. doi: 10.1007/s00394-017-1445-8.

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A.*, 1977; 74(12):5463–5467. doi: 10.1073/pnas.74.12.5463.

Santocchi E, Guiducci L, Fulceri F, et al. Gut to brain interaction in Autism Spectrum Disorders: a randomized controlled trial on the role of probiotics on clinical, biochemical and neurophysiological parameters. *BMC Psychiatry*., 2016; 16:183. doi: 10.1186/s12888-016-0887-5.

Sarkar C, Basu B, Chakroborty D, et al. The immunoregulatory role of dopamine: an update. *Brain Behav Immun.*,2010; 24(4):525-8. doi: 10.1016/j.bbi.2009.10.015.

Sassone L, Fidel R, Figueiredo L, et al. Evaluation of the microbiota of primary endodontic infections using checkerboard DNA-DNA hybridization. *Oral Microbiol Immunol.*, 2007; 22(6):390-7. doi: 10.1111/j.1399-302X.2007.00376.x.

Scheperjans F, Aho V, Pereira PAB, et al. Gut microbiota are related to Parkinson's disease and clinical phenotype. *Mov Disord*., 2015; 30(3):350-358. doi: 10.1002/mds.26069.

Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.*, 2009; 75:7537–7541. doi: 10.1128/AEM.01541-09.

Schloss P D. The effects of alignment quality, distance calculation method, sequence filtering, and region on the analysis of 16S rRNA gene-based studies. *PLoS Comput. Biol.*, 2010; 6:e1000844. doi:10.1371/journal.pcbi.1000844.

Sharpe MC, Archard LC, Banatvala JE, et al. A report Chronic fatigue syndrome: Guidelines for research. *J. R. Soc. Med*., 1991; 84(2):118–121.

Sheedy JR, Wettenhall RE, Scanlon D, et al. Increased D-lactic acid intestinal bacteria in patients with chronic fatigue syndrome. *In Vivo*, 2009; 23(4):621–628.

Shelokov A, Habel K, Verder E, et al. Epidemic neuromyasthenia; an outbreak of poliomyelitis like illness in student nurses. *N. Engl. J. Med.*, 1957; 257:345–355. doi: 10.1056/NEJM195708222570801.

Shen L, Weber CR, Raleigh DR, et al. Tight junction pore and leak pathways: A dynamic duo. Annu. *Rev. Physiol*., 2011; 73:283–309. doi: 10.1146/annurev-physiol-012110-142150.

Shukla SK, Cook D, Meyer J, et al. Changes in gut and plasma microbiome following exercise challenge in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). *PLoS One*, 2015; 10(12): e0145453. doi: 10.1371/journal.pone.0145453.

Singhrao SK, Harding A, Poole S, et al. Porphyromonas gingivalis periodontal infection and its putative links with Alzheimer's disease. *Mediators Inflamm.*, 2015; 137:357. doi:10.1155/2015/137357.

Skowera A, Stewart E, Davis ET, et al. Antinuclear autoantibodies (ANA) in Gulf War-related illness and chronic fatigue syndrome (CFS) patients. *Clin Exp Immunol.*, 2002; 129(2):354-8.doi:10.1046/j.1365-2249.2002.01912.x.

Slocum C, Kramer C, Genco CA. Immune dysregulation mediated by the oral microbiome: potential link to chronic inflammation and atherosclerosis. *Journal of internal medicine*, 2016; 280(1):114-28. doi: 10.1111/joim.12476.

Smith J, Fritz EL, Kerr JR, et al. Association of chronic fatigue syndrome with human leucocyte antigen class II alleles. *J Clin Pathol.*, 2005; 58(8):860-863. doi: 10.1136/jcp.2004.022681.

Sotzny F, Blanco J, Capelli E, et al. Myalgic Encephalomyelitis/Chronic Fatigue Syndrome - Evidence for an autoimmune disease. *Autoimmun Rev.*, 2018; 17(6):601-609. doi: 10.1016/j.autrev.2018.01.009.

Spigni G, Spina F, Poli A, et al. Molecular and Microbiological Insights on the Enrichment Procedures for the Isolation of Petroleum Degrading Bacteria and Fungi. *Front Microbiol*., 2018; 9:2543. doi: 10.3389/fmicb.2018.02543.

Srinivasan R, Karaoz U, Volegova M, et al. Use of 16S rRNA Gene for Identification of a Broad Range of Clinically Relevant Bacterial Pathogens. PLoS One., 2015; 10(2):e0117617. doi: 10.1371/journal.pone.0117617.

Stevanato Higuchi B, Rodrigues N, Ignácio Gonzaga M, et al. Intestinal Dysbiosis in Autoimmune Diabetes Is Correlated With Poor Glycemic Control and Increased Interleukin-6: A Pilot Study. *Front Immunol.*, 2018; 9:1689. doi: 10.3389/fimmu.2018.01689.

Stiemsma LT, Arrieta MC, Dimitriu PA, et al. Shifts in Lachnospira and Clostridium sp. in the 3-month stool microbiome are associated with preschool age asthma. *Clin Sci (Lond).*,2016; 130(23):2199-2207. doi:10.1186/s13223-016-0173-6.

Stolberg L, Rolfe R, Gitlin N, et al. D-Lactic acidosis due to abnormal gut flora: diagnosis and treatment of two cases. *N Engl J Med.*, 1982; 306(22):1344-8.doi:10.1056/NEJM198206033062207.

Strathdee F, Free A. Denaturing gradient gel electrophoresis (DGGE). *Methods Mol Biol.*, 2013; 1054: 145-57. doi: 10.1007/978-1-62703-565-1_9.

Sullivan A, Nord CE, Evengard B. Effect of supplement with lactic-acid producing bacteria on fatigue and physical activity in patients with chronic fatigue syndrome. *Nutr J.*, 2009; 8:4.doi: 10.1186/1475-2891-8-4.

Sullivan PF, Evengard B, Jacks A, et al. Twin analyses of chronic fatigue in a Swedish national sample. *Psychol Med*., 2005; 35(9):1327-1336. doi:10.1017/S0033291705005222.

Swain MG. Fatigue in chronic disease. *Clin Sci (Lond.)*, 2000; 99(1):1-8.

Swidsinski A, Ladhoff A, Pernthaler A, et al. Mucosal flora in inflammatory bowel disease. *Gastroenterology.*, 2002; 122(1):44–54. doi: 10.1053/gast.2002.30294.

Takahashi K, Nishida A, Fujimoto T, et al. Reduced Abundance of Butyrate-Producing Bacteria Species in the Fecal Microbial Community in Crohn's Disease. *Digestion.*, 2016; 93(1):59-65. doi: 10.1159/000441768.

Tan SZ, Begley P, Mullard G, et al. Introduction to metabolomics and its applications in ophthalmology. *Eye (Lond).*, 2016; 30(6):773–783. doi: 10.1038/eye.2016.37.

Tana C, Umesaki Y, Imaoka A, et al. Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. *Neurogastroenterol Motil.*, 2010; 22(5):512–9. e114-5. doi: 10.1111/j.1365-2982.2009.

Tap J, Derrien M, Törnblom H, et al. Identification of an Intestinal Microbiota Signature Associated With Severity of Irritable Bowel Syndrome. *Gastroenterology*, 2017;152(1):111–123.e8. doi: 10.1053/j.gastro.2016.09.049.

The Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature, 2012; 486(7402):207–214. doi: 10.1038/nature11234.

Thomas CM, Versalovic J. Probiotics-host communication modulation of signaling pathways in the intestine. *Gut Microbes.*, 2010; 1(3):148–63. doi:10.4161/gmic.1.3.11712

Thursby E, Juge N. Introduction to the human gut microbiota. *Biochemical Journal*, 2017; 474(11):1823-1836. doi: 10.1042/BCJ20160510.

Tizard IR. Macrophage cytophilic antibody in mice: effect of bacterial lipopolysaccharide on the uptake of immunoglobulins by mouse peritoneal cells. *Infect Immun.*, 1971; 3(3):472-7. doi: 10.1128/IAI.69.7.4342-4350.2001.

Turnbaugh PJ, Bäckhed F, Fulton L, et al. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host Microbe*., 2008; 3(4):213-23. doi:10.1016/j.chom.2008.02.015.

Turner JR. Intestinal mucosal barrier function in health and disease. Nat. Rev. *Immunol.*,2009; 9(11):799–809. doi: 10.1038/nri2653.

Ubeda C, Bucci V, Caballero S, et al. Intestinal microbiota containing Barnesiella species cures vancomycin-resistant Enterococcus faecium colonization. *Infect Immun.*, 2013; 81(3):965-73. doi: 10.1128/IAI.01197-12.

Ursell KL, Metcalf JL, Parfrey L. Defining the human microbiome. *Nutrition Reviews*, 2013; 70:38-44. doi: 10.1111/j.1753-4887.2012.00493.x.

Vaahtovuo J, Munukka E, Korkeamäki M, et al. Fecal microbiota in early rheumatoid arthritis. *J Rheumatol.*, 2008; 35(8):1500-5.

Vallone D, Picetti R, Borrelli E. Structure and function of dopamine receptors. *Neurosci Biobehav Rev.*, 2000; 24(1):125-32. doi: 10.1016/s0149-7634(99)00063-9.

Valour F, Sénéchal A, Dupieux C, et al. Actinomycosis: etiology, clinical features, diagnosis, treatment, and management. *Infect Drug Resist.*, 2014; 7:183-97. doi: 10.2147/IDR.S39601.

Vemuri R, Sylvia KE, Klein SL, et al. The microgenderome revealed: sex differences in bidirectional interactions between the microbiota, hormones, immunity and disease susceptibility. *Semin Immunopathol*., 2019; 41(2):265-275. doi: 10.1007/s00281-018-0716-7.

Venegas DP, De la Fuente MK, Landskron G, et al. Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. *Front Immunol.*, 2019; 10:277. doi: 10.3389/fimmu.2019.00277.

Vogel DY, Vereyken EJ, Glim JE, et al. Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status. *J. Neuroinflamm*., 2013; 10:35. doi: 10.1186/1742-2094-10-35.

Vogt NM, Kerby RL, Dill-McFarland KA et al. Gut microbiome alterations in Alzheimer's disease. *Sci Rep.*, 2017;7(1):13537. doi: 10.1038/s41598-017-13601-y.

Wade WG. The oral microbiome in health and disease. *Pharmacol Res*., 2013; 69(1):137-43. doi: 10.1016/j.phrs.2012.11.006.

Walsh CM, Zainal NZ, Middleton SJ, et al. A family history study of chronic fatigue syndrome. *Psychiatric Genetics*, 2001; 11 (3):123–8. doi:10.1097/00041444-200109000-00003.

Wang L, Christophersen C T, Sorich MJ, et al. Elevated fecal short chain fatty acid and ammonia concentrations in children with autism spectrum disorder. *Digestive Diseases and Sciences*, 2012; 57:2096–2102. doi:10.1007/s10620-012-2167-7.

Wang B, Jiang X, Cao M, et al. Altered Fecal Microbiota Correlates with Liver Biochemistry in Nonobese Patients with Non-alcoholic Fatty Liver Disease. *Sci Rep*., 2016; 6:32002. doi:10.1038/srep32002.

Wang T, Yu L, Xu C, et al. Chronic fatigue syndrome patients have alterations in their oral microbiome composition and function. *PLoS ONE*, 2018; 13(9): e0203503. doi: 10.1371/journal.pone.0203503.

Wang Y, Gao X, Ghozlane A, et al. Characteristics of Faecal Microbiota in Paediatric Crohn's Disease and Their Dynamic Changes During Infliximab Therapy. *J Crohns Colitis*., 2018; 12(3):337-346. doi: 10.1093/ecco-jcc/jjx153.

Ware JE Jr, Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. Med Care., 1992; 30(6):473-83.
Wexler HM. Bacteroides: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev*., 2007; 20(4):593-621. doi: 10.1128/CMR.00008-07.

Wong JM, de Souza R, Kendall CW, et al. Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol.*, 2006; 40(3):235-43. doi: 10.1097/00004836-200603000-00015.

Wu GD, Chen J, Hoffmann C, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science., 2011; 334(6052):105-8. doi: 10.1126/science.1208344.

Wu S, Lim KC, Huang J, et al. Bacteroides fragilis enterotoxin cleaves the zonula adherens protein, E-cadherin. *Proc Natl Acad Sci U S A.*, 1998; 95(25):14979-84. doi: 10.1073/pnas.95.25.14979.

Wu S, Rhee KJ, Albesiano E, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat Med*., 2009; 15:1016–22. doi:10.1038/ nm.2015.

Wu X, Ma C, Han L, et al. Molecular characterisation of the faecal microbiota in patients with type II diabetes. *Curr Microbiol.*, 2010; 61(1):69–78. doi: 10.1007/s00284-010-9582-9.

Wylie KM, Truty RM, Sharpton TJ, et al. Novel Bacterial Taxa in the Human Microbiome. *PLoS One*., 2012; 7(6): e35294. doi: 10.1371/journal.pone.0035294.

Yeung DF, Parsa A, Salh B. A Case of Rothia dentocariosa Bacteremia in a Patient Receiving Infliximab for Ulcerative Colitis. *Am J Gastroenterol.,* 2014; 109(2): 297-8.doi:10.1038/ajg.2013.366.

Yu Z, Morrison M. Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.*, 2004; 70(8):4800–4806. doi:10.1128/AEM.70.8.4800-4806.2004.

Zhang R, Miller RG, Gascon R, et al. Circulating endotoxin and systemic immune activation in sporadic amyotrophic lateral sclerosis (sALS). *J Neuroimmunol.*, 2009; 206(1-2):121-4. doi:10.1016/j.jneuroim.2008.09.017.

Zhang YJ, Li S, Gan RY. Impacts of Gut Bacteria on Human Health and Diseases. *International Journal of Molecular Sciences.*, 2015; 16(4):7493-7519. doi: 10.3390/ijms16047493.

Zhu S, Liu S, Li H, et al. Identification of Gut Microbiota and Metabolites Signature in Patients With Irritable Bowel Syndrome. *Front Cell Infect Microbiol.*, 2019; 9:346. doi:10.3389/fcimb.2019.00346. doi: 10.3389/fcimb.2019.00346.

Zhuang ZQ, Shen LL, Li WW, et al. Gut Microbiota is Altered in Patients with Alzheimer's Disease. *J Alzheimers Dis.*, 2018; 63(4):1337-1346. doi: 10.3233/JAD-180176.

Zhul X, Han Y, Du J, et al. Microbiota-gut-brain axis and the central nervous system. *Oncotarget*., 2017; 8(32):53829-53838. doi: 10.18632/oncotarget.17754.

Zijnge V, van Leeuwen MBM, Degener JE, et al. Oral Biofilm Architecture on Natural Teeth. *PloS one*., 2010; 5(2): e9321. doi: 10.1371/journal.pone.0009321.

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