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High-throughput 16S rRNA gene sequencing reveals alterations of intestinal microbiota in myalgic encephalomyelitis/chronic fatigue syndrome patients☆

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ABSTRACT

Human intestinal microbiota plays an important role in the maintenance of host health by providing energy, nutrients, and immunological protection. Intestinal dysfunction is a frequent complaint in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients, and previous reports suggest that dysbiosis, i.e. the overgrowth of abnormal populations of bacteria in the gut, is linked to the pathogenesis of the disease.

We used high-throughput 16S rRNA gene sequencing to investigate the presence of specific alterations in the gut microbiota of ME/CFS patients from Belgium and Norway. 43 ME/CFS patients and 36 healthy controls were included in the study. Bacterial DNA was extracted from stool samples, PCR amplification was performed on 16S rRNA gene regions, and PCR amplicons were sequenced using Roche FLX 454 sequencer.

The composition of the gut microbiota was found to differ between Belgian controls and Norwegian controls: Norwegians showed higher percentages of specific Firmicutes populations (*Roseburia*, *Holdemania*) and lower proportions of most Bacteroidetes genera. A highly significant separation could be achieved between Norwegian controls and Norwegian patients: patients presented increased proportions of *Lactonifactor* and *Alistipes*, as well as a decrease in several Firmicutes populations. In Belgian subjects the patient/control separation was less pronounced, however some abnormalities observed in Norwegian patients were also found in Belgian patients.

These results show that intestinal microbiota is altered in ME/CFS. High-throughput sequencing is a useful tool to diagnose dysbiosis in patients and could help designing treatments based on gut microbiota modulation (antibiotics, pre and probiotics supplementation).

1. Introduction

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a debilitating disease with a worldwide prevalence of 0.4–1%. According to the definition of Fukuda et al. [1], ME/CFS patients present a clinically evaluated, unexplained fatigue accompanied by at least four of the following symptoms: memory impairment, sore throat, tender lymph nodes, muscle or joint pain, headaches, unrefreshing sleep, post-exertional malaise. A number of conditions such as malignancies and certain viral infections exclude a diagnosis of ME/CFS. The pathogenesis of the disease is still poorly understood, but probably involves multiple factors such as infections, stress, and neuroendocrine impairments [2].

A number of observations suggest that gastro-intestinal dysfunctions also contribute to the onset and/or maintenance of the disease [3]. There is a striking lifetime rate (92%) of irritable bowel syndrome (IBS) among ME/CFS patients [4]; co-occurrence of ME/CFS and IBS is associated with increased plasma levels of pro-inflammatory cytokines such as IL-6, IL-8, IL-1β and TNFα [5], a systemic inflammatory situation which may in part be due to the translocation of commensal bacteria resulting from an increased

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intestinal permeability [6]. Gut inflammation and loss of mucosal integrity may therefore contribute to the pathogenesis of the disease.

A growing body of literature is linking intestinal and immune dysfunctions with alterations in the composition of the gut microbiota. Intestinal bacteria play major roles in breakdown and synthesis of nutrients, prevention of infection by pathogenic organisms, regulation of mucosal and systemic immunity, and even CNS function (the "gut-brain" axis), influencing emotional behaviors such as anxiety and depression. Overgrowth or deficiencies of specific bacterial populations have been associated with various disorders, including IBS, Crohn's disease, allergies, autoimmune diseases, diabetes and cancer [7–11].

Alterations in gut microbiota composition have already been reported for ME/CFS. In 2003 Logan et al. [12] hypothesized that low levels of bifidobacteria and small intestinal bacterial overgrowth (SIBO) could result in immune dysfunctions in ME/CFS patients. Using culture-based assays, Sheedy et al. [13] observed significantly increased proportions of D-lactic acid-producing *Enterococcus* and *Streptococcus* spp. in faecal samples of ME/CFS patients. Excess D-lactic acid production could contribute to mitochondrial dysfunction, but also to neurocognitive impairments in patients, since D-lactic acidosis is known to affect CNS function. The link between intestinal microbiota and cognitive problems was further supported by a study reporting a positive effect of probiotic supplementation (*Lactobacillus casei*) on emotional and anxiety symptoms in ME/CFS patients [14]. In another study, administration of probiotic strains (*Lactobacillus paracasei*, *Lactobacillus acidophilus*, *Bifidobacterium lactis*) also resulted in a significant improvement of neurocognitive functions, but not of fatigue and physical activity scores [15]. These results warranted further studies on gut microbiota composition in ME/CFS.

Recently, new technologies based on metagenomics/high-throughput sequencing have been developed and successfully applied to the analysis of the complex bacterial ecosystem of the gut. These new analytical approaches usually involve DNA extraction (from stool samples or biopsies) and amplification of 16S ribosomal RNA gene followed by high-throughput sequencing. By sequence analysis, several thousands of bacteria can be identified in each sample. Whereas previous culture-based assays could only address cultivable organisms (which only represent a small proportion of the hundreds of species inhabiting the gut), sequencing-based molecular technologies can give a comprehensive overview of the gut microbiota, including the four major phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, and have already revealed associations between microbiota composition and disorders like obesity, IBS, allergies, and autism [16–19].

In this study, we used a high-throughput sequencing approach to investigate the microbiota composition of stool samples from Belgian and Norwegian ME/CFS patients. Significant alterations in microbiota composition were observed in patients compared to matched healthy controls, suggesting an involvement of intestinal dysbiosis in the pathogenesis of the disease.

2. Patient selection, methods

2.1. Patient selection

The following subjects were recruited for the study: Belgian healthy controls ($n = 19$, 15 women and 4 men, mean age 41 $\pm$ 12.6, median age 41), Belgian patients ($n = 18$, 15 women and 3 men, mean age 38.5 $\pm$ 13, median age 37), Norwegian healthy controls ($n = 17$, 14 women and 3 men, mean age 45 $\pm$ 19, median age 49), Norwegian patients ($n = 25$, 22 women and 3 men, mean age 41 $\pm$ 12.5, median age 42). All subjects were Caucasians. Patients were diagnosed for CFS according to the clinical criteria of Fukuda et al. [1]. 14 out of the 18 Belgian patients, and 20 out of the 25 Norwegian patients, presented moderate to severe intestinal symptoms (pain, diarrhea, bloating). Illness duration was 7 $\pm$ 4 years for the Belgian patients, 12 $\pm$ 9 years for the Norwegian patients. The subjects had not been taking antibiotics or probiotics for four weeks prior to sample collection; they gave written informed consent to the study.

2.2. Sample collection, DNA extraction

Samples of homogenized stool were collected and directly mixed with 2 ml of Aquastool solution (Multi Target Pharmaceuticals, Salt Lake City, USA). Aquastool stabilizes the sample and prevents any modification of microbiota composition, even in samples stored for up to four days at room temperature (information provided by the manufacturer). For long term storage samples were kept at −20 °C. For DNA extraction, the stool sample was centrifuged and washed twice with PBS. After centrifugation, the supernatant was discarded and the pellet resuspended in 2 ml of TE. One milliliter of the suspension was mixed with 5 mg of Lysozyme (Sigma) and glass beads (Sigma) and incubated at 37 °C for 1 h and 30 min in a thermomixer (1400 rpm). After adding 32 µl of proteinase K (20 mg per ml, Roche) and 400 µl of SDS (10%), the samples were incubated at 55 °C for 1 h. Samples were further extracted using the DNeasy Blood and Tissue kit (Qiagen) following manufacturer instructions.

2.3. PCR amplification, high-throughput sequencing

16S rRNA genes were amplified using the primers AGGATTAGATACCCTGGTA and CRRCACGAGCTGACGAC targeting the V5 and V6 hypervariable 16S rRNA regions [20]. For each sample, a PCR mix of 100 µl was prepared containing 1× PCR buffer, 2 U of KAPA HiFi Hotstart polymerase blend and dNTPs (Kapabiosystems), 300 nM primers (Eurogentec, Liege, Belgium), and 60 ng gDNA. Thermal cycling consisted of initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 98 °C for 20 s, annealing at 56 °C for 40 s, and extension at 72 °C for 20 s, with a final extension of 5 min at 72 °C. Amplicons were visualized on 1% agarose gel using GelGreen Nucleic Acid gel stain in 1× TAE (Biotium) and were cleaned using the Wizard SV Gel and PCR Clean-up System (Promega) according to the manufacturer's instructions.

Amplicon DNA concentrations were determined using the Quant-iT PicoGreen dsDNA reagent and kit (Life Tech, Carlsbad, USA) following the manufacturer's instructions. After quantification, the DNA was pooled and purified using Agencourt Ampure XP Purification systems according to the manufacturer's instructions (Agencourt Biosciences Corporation, Beckman Coulter, USA). Pyrosequencing was carried out following the manufacturer instructions on a 454 Life Sciences Genome Sequencer FLX instrument (Roche) using titanium chemistry.

The sequences were assigned to samples according to sample-specific barcodes. Sequences were then checked for the following criteria: (i) almost perfect match with barcodes and primers; (ii) length of at least 240 nucleotides (barcodes and primers excluded); (iii) no more than two undetermined bases (denoted by N). By "almost perfect match", we mean that one mismatch/deletion/insertion is allowed in the barcode, idem for the primer. Each sequence originating from pyrosequencing and passing QC was assigned to a family by the RDP classifier (v 2.1) with CE $>$ 80%. The Shannon and Chao richness estimates were done through the Mothur package [21].

2.4. Statistical analysis

Data were analyzed using Mann–Whitney test and linear discriminant analysis. The Mann–Whitney hypothesis test

compared the shift in distribution between pairs of groups. Linear discriminant analysis (LDA) was applied to map the discriminatory performance of sets of bacteria with respect to the population samples. The first and second discriminant function scores were used for display [22–24]. The significance of LDA was tested using the associated MANOVA Wilk's Lambda test. All results were deemed significant if the *p*-value was below 0.05, the level of significance adopted.

The data analysis was performed using SPSS (Statistical Package for the Social Sciences, IBM, Armonk, USA) version 14.0.

3. Results

3.1. Number of reads per sample, classification of assigned sequencing by phylum, family and genus

On average, 6000–7000 reads were obtained per sample. For all samples, at least 98% of reads could be assigned to a specific phylum (usually more than 99% assignation). Represented phyla were Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria; in some samples bacteria belonging to the phyla Euryarchaeota, Fusobacteria, Verrucomicrobia or Lentisphaerae could be detected but in very small proportions (not more than 0.02% of total reads).

As expected, and due to the stringent criteria used for sequence analysis and classification, the number of reads that could be assigned with certainty to a specific family or a specific genus was lower (60–80% assignation at the family level, 30–60% assignation at the genus level).

129 Different bacterial genera could be identified from the 79 samples included in the study. A majority of these however were present only in a few samples (sometimes just one or two subjects, in a very small proportion).

Genera represented in almost all subjects (there were always exceptions, in both controls and patients) were *Anaerostipes*, *Bacteroides*, *Blautia*, *Bifidobacterium*, *Butyricicoccus*, *Coprococcus*, *Dorea*, *Faecalibacterium*, *Oscillibacter*, *Roseburia*, *Ruminococcus*, *Subdoligranulum*. Among those, the usually dominant genera were *Bifidobacterium* (very variable, from 0 to more than 50%), *Blautia* (15–60%), *Coprococcus* (2–3% up to 30%), *Dorea* (usually 5–15%), *Faecalibacterium* (usually 5–20%), *Roseburia* (up to 45% in some samples).

Many genera were characterized by a strong variability in term of representativity, being absent in some samples but forming a significant percentage of the total bacterial population in others. *Lactonifactor* was usually low or absent, but could reach more than 7% in some samples. *Clostridium* was anywhere between 0 and 15%. Usually below 5%, *Streptococcus* reached 15%, 44% and 60% in three subjects. Other genera showing high variability were *Dialister*, *Colinsella*, *Prevotella*, *Asaccharobacter*, *Alistipes*.

3.2. Geographical origin of the sample and microbiota composition

There were strong variations of diversity index between individuals: non-parametric Shannon index (OTU cutoff 0.10) varied from 2.6 to 3.54 in Belgian controls, from 2.2 to 3.7 in Norwegian controls. However, average diversity index did not differ significantly between the four populations.

Discriminant function analysis was performed using percentages of the different bacterial genera as variables. The four groups of samples clustered separately, and the separation was statistically significant ($p = 0.022$). ME/CFS patients therefore appear to have a different gut microbiota composition than healthy controls; differences could also be seen between the healthy controls of the two different geographical origins (Fig. 1).

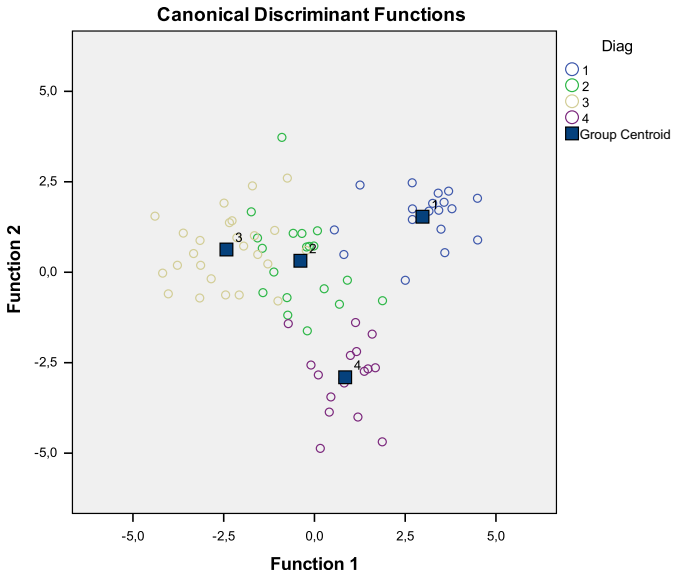

Fig. 1. Discriminant function analysis separates the sample into distinct clusters. 1: Belgian patients; 2: Belgian controls; 3: Norwegians patients; 4: Norwegian controls.

Interestingly Belgian and Norwegian patients diverged from their corresponding controls in two different ways: alterations seen in Belgian patients may therefore at least partially differ from the alterations seen in Norwegian patients.

The Mann–Whitney test was used to compare Belgian controls with Norwegian controls. Norwegian controls presented a significantly lower percentage of Bacteroidetes bacteria ($p = 0.026$) and a 3-fold increase of the Firmicutes/Bacteroidetes ratio.

At the genus level, Norwegians showed higher percentages of specific Firmicute populations compared to Belgians: an average 1.7-fold increase of *Roseburia* ($p = 0.048$), and a 3-fold increase of *Holdemania* ($p = 0.008$). They showed lower proportions of several Bacteroidetes genera: three times less *Bacteroides* ($p = 0.015$), five times less *Alistipes* ($p = 0.009$), four times less *Prevotella* ($p = 0.002$) (Table 1).

3.3. Alterations of intestinal microbiota in Norwegian patients

Discriminant function analysis achieved a highly significant separation ($p < 0.001$) between Norwegian controls and Norwegian patients (Fig. 2). Variations in sub-populations of the Firmicutes phylum (namely relative proportions of the genera *Moryella*, *Ethanoligenens*, *Ruminococcus*, *Eubacterium*, *Holdemania* and *Turicibacter*) were main contributing factors of this separation. In patients, populations of *Ethanoligenens* and *Moryella* are increased, populations of *Ruminococcus*, *Eubacterium*, *Holdemania* and *Turicibacter* are decreased.

Between Norwegian controls and Norwegian patients, the most significant differences obtained by the Mann–Whitney test were for the Firmicutes genera *Holdemania* (50-fold decrease, $p = 0.0001$), *Lactonifactor* (20-fold increase, $p = 0.003$), *Syntrophococcus* (2.5-fold decrease, $p = 0.015$), but also for the Bacteroidetes *Alistipes* (3.8-fold increase, $p = 0.013$) (Table 2).

3.4. Alterations of intestinal microbiota in Belgian patients

In Belgian subjects the patient/control distinction was less pronounced (Fig. 3). The separation obtained by discriminant function analysis was not significant (although borderline, $p = 0.072$). Interestingly however, some of the variables which

Table 1
Variation of specific bacterial genera in Norwegian controls vs. Belgian controls. Levels of significance: $^{*}p < 0.05$, $^{**}p < 0.01$.

Norwegian controls vs. Belgian controls	
Genus	**Increase/Decrease**
Roseburia	x1,7*
Holdemania	x3**
Bacteroides	x0.36*
Alistipes	x0.2**
Barnesiella	x0.2**
Parabacteroides	x0.26**
Prevotella	x0.025**

Table 2
Variation of specific bacterial genera in Norwegian patients vs. Norwegian controls. Levels of significance: $^{*}p < 0.05$, $^{**}p < 0.01$.

Norwegian patients vs. Norwegian controls	
Genus	**Increase/Decrease**
Roseburia	x0.54*
Syntrophococcus	x0.4*
Lactonifactor	x20**
Holdemania	x0.02**
Dialister	x0.6*
Alistipes	x3,8*

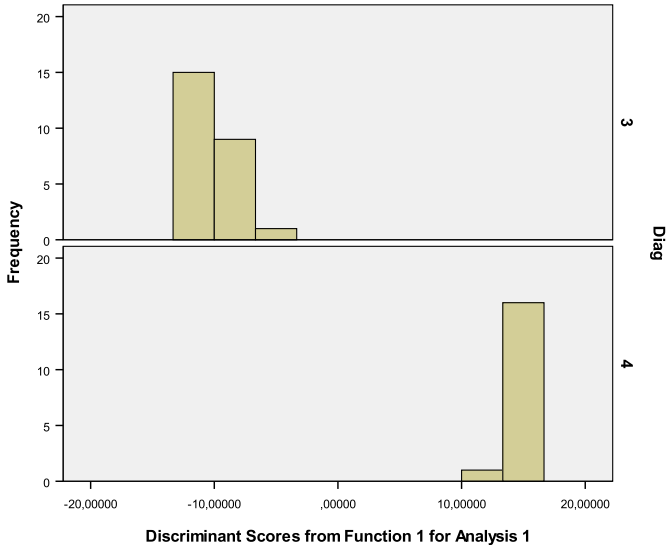

Fig. 2. Discriminant function analysis allows excellent separation of the two populations (3: Norwegian patients, 4: Norwegians controls), with no overlap.

contributed most to this separation were the same as the ones which could also discriminate Norwegian patients from Norwegian controls: proportions of *Ethanoligenens*, *Ruminococcus*, *Eubacterium* were again main contributors in the discriminant function (together with *Blautia*, *Lactobacillus* and *Megasphera*).

Using the Mann–Whitney test, only two variables were found to differ significantly between Belgian controls and Belgian patients: the percentage of *Asaccharobacter*, a member of the Actinobacteria

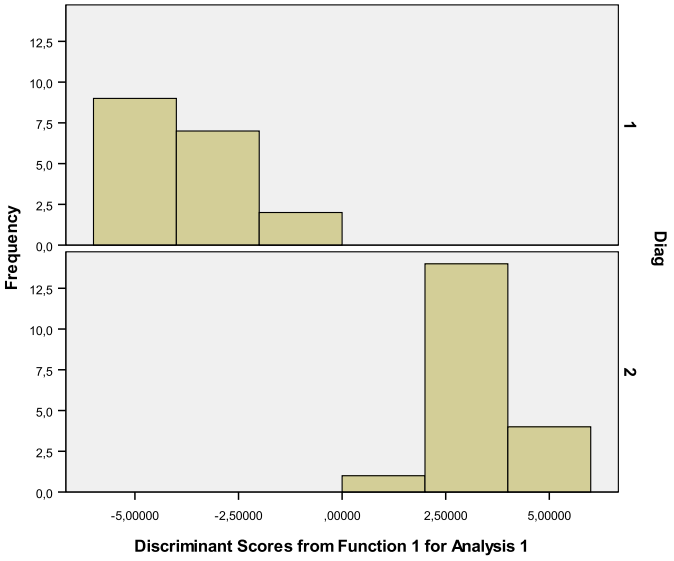

Fig. 3. Discriminant function analysis separates the two populations (1: Belgian patients, 2: Belgians controls), but the separation is less significant than for the Norwegian subjects.

phylum (4-fold decrease, $p = 0.041$), and also, remarkably, the percentage of *Lactonifactor* which was again strongly increased in patients (45-fold increase, $p = 0.006$) (Table 3).

4. Discussion

Bacteria living in the intestine can exert a critical influence on host health by synthesizing nutrients and vitamins, producing beneficial or toxic metabolites, inhibiting microbial and viral pathogens, detoxifying food, and also by contributing to the development of an optimally functioning immune system. 70% of all immune cells are located in the gastro-intestinal tract, and their interactions with bacterial antigens at the mucosal surface contribute to the regulation of major immune mechanisms such as tolerance, allergy and inflammation [8].

Acquisition of the intestinal microbiota occurs mostly during the first two–three years of life and is influenced by many factors: environmental factors such as vaginal vs. Cesarean delivery, breast vs. bottle feeding, lifestyle, use of antibiotics, but also host factors such as immune system function and genetic background [8,25]. Host factors are probably largely responsible for the resilience of the gut microbiota composition which, once established in the young child, seems to remain relatively stable throughout life. In the long term however, modifications of dietary habits can affect the composition of the gut microbiota; the so-called "Western diet", high in sugar and fat, seems to favor the growth of Firmicutes including *Clostridium*, *Eubacterium*, *Enterococcus* spp., and decrease several *Bacteroides* spp. Consumption of refined sugars mediates the overgrowth of opportunistic *Clostridium difficile* and *Clostridium perfringens*, whereas complex carbohydrates favor beneficial *Bifidobacteria* spp. [26].

These modifications are suspected to affect the host inflammatory and metabolic responses, and disruption of intestinal microbiota equilibrium has been associated with several diseases. In most cases however it is not clear how specific bacterial populations contribute to pathogenesis. Defining a "normal" intestinal microbiota is probably not possible: adult gut microbiota composition varies dramatically from one individual to another, with differences in the ratios of dominant phyla, as well as in the presence and proportions of multiple genera and species. Depending on host genetics and dietary factors, a specific microbiota may be symbiotic or commensal in some individuals, but have detrimental activities, and predispose to certain diseases, in other people.

The large majority of all sequences identified in our subjects (controls and patients) belonged to one of the four phyla Actinobacteria, Bacteroidetes, Firmicutes or Proteobacteria, which is in agreement with previous reports from human gut microbiota studies [27]. However the relative proportions of these major phyla differed from one individual to another: geographical origin of the sample had an influence on the Firmicutes/Bacteroidetes ratio which was significantly higher in Norwegian samples than in Belgian samples.

This difference may be due to genetic and/or dietary factors. A recent study, using the same methodology as ours, found that Actinobacteria and Bacteroidetes were more abundant in African children's microbiota, whereas in Italian children, Firmicutes was the dominant phyla [28]. Bacteroidetes bacteria like *Xylanibacter* and *Prevotella* are able to ferment plant polysaccharides (xylan, cellulose) which are present in the rural African diet but much less in the food consumed in industrialized countries; it was hypothesized that the dominance of Bacteroidetes in African children was an adaptation to the available diet, allowing individuals to maximize energy intake from fibre-rich food.

Although there may be differences between Belgian and Norwegian diets, the differences observed in our study may also result from the influence of genetic background. Any gene involved in mucosal immunity regulation, production of mucin or antibacterial defensins by gut epithelial cells, could potentially influence the composition of the microbiota, and further studies should investigate how polymorphisms in such genes can affect the final composition of the gut ecosystem. A number of genes involved in mucosal immunity such as IRAK3, IL22, IFNG, ATG16L, TLRs and CARD15/NOD2 have already been related to alterations of gut microbiota composition [25,29].

Our study has revealed alterations of gut microbiota composition in ME/CFS patients, which were more significant in the Norwegian subjects. Norwegian patients differ from their matched controls by showing decreased percentages of several Firmicutes sub-populations (*Roseburia*, *Syntrophococcus*, *Holdemania*, *Dialister*), a strong 20-fold increase of *Lactonifactor*, and a 3.8-fold increase of the Bacteroidetes genus *Alistipes*.

Syntrophococcus and *Holdemania* are poorly characterized genera, it is difficult to discuss how their decrease in patients can be associated with ME/CFS pathogenesis. *Roseburia* is a major butyrate-producing genus; as such these bacteria contribute to colon epithelium health, production of energy, and protection against gut inflammation [30]. *Dialister* is best known clinically for its association with periodontal disease [31], however its presence in the gut may not be detrimental; actually a decrease of *Dialister invisus* in fecal samples from patients with Crohn's disease has already been reported [32]. Increase of *Alistipes* can also be related to gut inflammation: higher levels of *Alistipes* taxa are associated with greater frequency of abdominal pain in irritable bowel syndrome (IBS) patients [33], and actually the bacteria was first described as a *Bacteroides*-related, bile-resistant rod often isolated from appendicitis tissue [34].

Alterations of gut microbiota observed in Norwegian patients are therefore consistent with an increased intestinal inflammation, which is suspected to occur in ME/CFS patients [3–6].

It is of course notable that the Firmicutes/Bacteroidetes ratio did not decrease to such an extent in the Belgian patients. This may reflect a different disease pathogenesis, but may also simply result from the differences already existing in the control populations. Firmicute populations are already low in the Belgian controls; whether this predisposes Belgians to gut inflammation, or simply represents a healthy adaptation to different genetic background and diet, is unclear.

Norwegian patients are slightly older than Belgian patients (mean age 41 vs. 38.5) and tend to have been sick for a longer period of time (12 ± 9 vs. 7 ± 4 years). This longer illness duration

Table 3
Variation of specific bacterial genera in Belgian patients vs. Belgian controls. Levels of significance: $^{*}p < 0.05$, $^{**}p < 0.01$.

Belgian patients vs. Belgian controls	
Genus	**Increase/Decrease**
Lactonifactor	x45**
Asaccharobacter	x0.25*

could have partially explained the stronger alterations seen in Norwegian patients, however statistical analysis did not reveal any significant correlation between microbiota composition and time since disease onset.

Recently Arumugam et al. [35] reported the results of a metagenomic study involving 39 individuals from different geographical origins. They found that samples could be grouped into three distinct clusters (called enterotypes), driven by species composition. The dominant species in these three clusters were *Ruminococcus*, *Prevotella* and *Bacteroides*, respectively.

Although Arumugam et al. did not observe any relation between the geographical origin of the samples and their grouping into a specific cluster, it is notable that in our study, *Bacteroides* and *Prevotella* were two of the species which differentiated the Norwegian controls from the Belgian controls. None of the three species, however, was involved in the separation between controls and patients, so there is no evidence that disease could be associated with a shift from one specific enterotype to another.

A remarkable similarity between Norwegian and Belgian patients was the strong, specific increase of *Lactonifactor* (20-fold in Norwegian patients, 45-fold in Belgian patients). *Lactonifactor* is a Firmicute from the *Clostridiaceae* family. Although this genus is not very well characterized, it is known to be involved in the conversion of lignan phytoestrogens into bioactive enterodiol (ED) and enterolactone (EL) [36,37]. In Belgian patients, the increase of *Lactonifactor* was paralleled by a decrease of the Actinobacteria *Asaccharobacter*. The association of these two alterations may be significant, since *Asaccharobacter* is also involved in the metabolism of phytoestrogens, being able to convert isoflavones like daidzein into equol [38,39]. One could hypothesize that intestinal dysbiosis contributes to ME/CFS through altered production of estrogen receptor (ER) agonists/antagonists, which could interact with estrogen-dependent pathways. Obviously the predominance of women among patients, the fact that disease symptoms change upon e.g. puberty, pregnancy and menopause, has long suggested an involvement of hormones in ME/CFS and actually ERβ, the preferred receptor of equol, is down-regulated in patients [40,41]. Disruption of estrogen signaling is therefore an interesting hypothesis, quite consistent with some dysfunctions observed in ME/CFS. It could be related, for instance, with immune dysregulation since isoflavones and more specifically equol induce expression of antioxidant enzymes, and exert an anti-inflammatory action by inhibiting the production of IL-6, TNF-α and inducible nitric oxide synthase in LPS-stimulated macrophages [42,43].

Another potentially relevant aspect of phytoestrogens and their metabolites is their capacity to regulate vitamin D receptor (VDR) activity, and vitamin D synthesis. Similar to 17β-estradiol, phytoestrogens can induce expression of VDR in gut epithelial cells; they also regulate several cytochrome P450 enzymes which are integral part of vitamin D synthesis pathways [44]. The vitamin D system is critical for maintenance of gut mucosal immunity, and several reports have shown a link between vitamin D, VDR, and inflammatory intestinal diseases [45,46]. VDR-mediated signaling can antagonize TNF-α, IL-17, IL-6, IL-1 in macrophages and dendritic cells and prevent development of inflammatory bowel disease. Vitamin D has also been shown to modulate T-cell antigen receptor and induce FoxP3, which is critical for prevention of mucosal inflammation [47,48].

A number of observations suggest an alteration of VDR function in ME/CFS [49]. The potential interaction of gut bacteria metabolites with the regulation mechanisms of VDR is therefore an interesting mechanism which could explain this important aspect of the disease.

Our study has revealed significant alterations of intestinal microbiota in ME/CFS patients. Metagenomic high-throughput sequencing has only recently been applied to the analysis of gut microbiota; as a result, most of the bacterial genera identified by this technique are still poorly characterized, and a lot has still to be learned about their influence on gut function and human health. However, given the state of the knowledge, the various alterations observed seem consistent with the hypothesis of an increased intestinal inflammation in ME/CFS; they also suggest a possible mechanism where bacterial metabolites could contribute to the disease by interfering with estrogen receptor and VDR pathways. Further experiments are being designed to test these hypotheses; for instance the combination of metagenomic microbiota analyses with metabolomic approaches (broad range analysis of metabolites of human and bacterial origin present in stool, urine or plasma samples) could help establish some links between the presence of specific bacterial populations, and the modification of human physiological mechanisms.

Like most gut microbiota studies published so far, the present study has been analyzing bacterial populations in stool samples. Some authors have suggested that stool bacteria are merely representative of the luminal microbiota, which is not necessarily identical to the bacterial populations living in close contact with the gut mucosa [50]. Mucosa-associated populations, however, differ from one place of the digestive tract to another. The stool sample may therefore provide a more representative view of the global microbiota than a single punch biopsy.

A question of major importance is of course the causal relationship between gut microbiota alterations and disease onset; this point still has to be clarified. Is altered gut microbiota a pre-existing, causative or at least predisposing factor for the disease? Or do microbiota alterations occur as a consequence of the disease, triggered by stress, immune dysfunctions or pathogen infections in the intestine? For instance, we and others have reported the presence of persistent viral infections (enterovirus, parvovirus B19, herpesviruses) in the gastro-intestinal tract of ME/CFS patients [51,52]. These viruses could reactivate as a consequence of altered mucosal immunity caused by bacterial dysbiosis (replication of herpesviruses is favored by inflammation); but since some of these viruses have potent immunosuppressive effects, the reverse mechanism where a viral infection causes a change in mucosal immunity, which in turn leads to gut microbiota alterations, is also possible. Longitudinal studies examining the evolution of microbiota over a certain period of time, through relapses and remission, or before and after treatments, could already give a partial answer. Further studies will have to clarify the complex relationships between bacterial microbiota, viral infections, host immune system and genetic background.

5. Conclusions

High-throughput rRNA gene sequencing proved to be a useful tool to diagnose dysbiosis in ME/CFS patients. Our results suggest new pathogenesis mechanisms for ME/CFS, but also open new possibilities regarding the design of treatments based on gut microbiota modulation using antibiotics, pre and probiotics supplementation.

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