ORIGINAL ARTICLE

A Study of the Gut Microbiome in Egyptian Patients with active Crohn's Disease

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ABSTRACT

Background*: The pathogenesis of Crohn's disease (CD) is multifactorial. Gut microbiota alteration (dysbiosis) which is a shift in the microbial populations inhabiting the gut, is considered a novel factor involved in the pathogenesis of CD. The aim of the work is to describe and study the change in the gut microbiome profile of Egyptian CD patients.* ***Methodology:*** *The study included twenty-four Egyptian individuals with active CD. The activity of the CD was determined according to a combination of clinical and endoscopy indices (the Mayo Clinic index and the Disease Activity Index). Stool specimens were subjected to microbiome analysis using the quantitative SYBR Green real-time PCR method.* ***Results****: Patients with CD showed signs of significant dysbiosis, as indicated by a statistically significant reduction in Firmicutes and Ruminococcus abundances relative to the control group (P=0.001, P=0.016), respectively. Patients with CD had a significantly lower Firmicutes/ Bacteroidetes (F/B) ratio than the control group (P=0.003).Furthermore, when compared to the control group, the F. prausnitzii, Lactobacilli (P≤ 0.001), and Bifidobacteria (P=0.001) levels in the CD patients were statistically significantly lower. Regarding the Prevotella/Bacteroides ratio (P/B), there were no statistically significant differences seen between the CD patients and the controls.* ***Conclusion:*** *The current study revealed alterations in the gut microbiome of CD patients, compared with healthy controls. This could help identify the gut microbiota and particular bacterial modifications that can be targeted for CD treatment.*

INTRODUCTION

Any part of the gastrointestinal (GI) tract can be affected by Crohn's disease (CD), a subtype of inflammatory bowel disease (IBD), which is a complex, severe, spontaneous, evolving, and destructive condition with an unknown cause.[1] An abnormal immune response is the consequence of the interplay between host genetics, the immune system, the gut microbiota, and environmental factors, that causes intestinal inflammation, which is part of the multifactorial etiology of CD.[2] The gut microbiota is crucial from infancy and has a significant impact in physiological processes such as the immune system's maturation, intestinal homeostasis, behavior, and host metabolism.[3] Dysbiosis, or a disturbance in the gut microbiome, is linked to gastrointestinal and metabolic disorders such as IBD, which includes CD and ulcerative colitis (UC).[4]

The bulk of the gut microbiota in healthy humans is classified into four phyla: Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes, according to molecular analysis.[5] *A significant member of the phylum Firmicutes, Faecalibacterium prausnitzii (F. prausnitzii) is* one of the primary butyrate-producing bacteria in the healthy human microbiome.[6] The ability to promote the expression of IL-10, an anti-inflammatory cytokine, has been linked to butyrate synthesis. Moreover, it has been found that *F. prausnitzii* is a potent inducer of regulatory T cells that secrete IL-10.[7] Normal gut microbiota content directly influences the balance of Th17 and Treg cells, which are known for their pro- and anti-inflammatory cytokine production. This equilibrium is important for maintaining intestinal homeostasis in the host.[8-9] By promoting the manufacture of mucin and tightening the connections between epithelial cells, butyric acid reduces inflammation and leaky gut syndrome.[10] Bacteriocins, short-chain fatty acids, and lactic acid are examples of bactericidal acidic compounds produced by lactobacilli and Bifidobacteria. These fatty acids have a role in both reducing inflammation and promoting the creation of mucus.[11]

According to reports, there is a drop in proinflammatory microorganisms and an increase in anti-inflammatory ones in the gut dysbiosis of IBD patients. This phenomenon explains the disturbed and elevated production of proinflammatory cytokines observed in CD.[12]

People who have IBD have weaker mucus layers that allow luminal bacteria to penetrate the submucosal layers, triggering proliferative and inflammatory processes. Subsequently, inflammatory injury-induced mucosal deterioration causes the submucosa to be vulnerable to more bacteria, creating a vicious cycle of positive feedback between mucosal damage and antigenic exposure.[13]

Finding out how the CD microbiome has been altered may be crucial for future studies on the pathophysiology of the illness and for creating plans for therapy and prevention that rely on the microbiome. The purpose of the current investigation was to describe the profile of the gut microbiota. of CD patients in light of these data and associated factors.

METHODOLOGY

Patients

The study included 24 Egyptian patients with active CD who were enrolled from the Gastroenterology Outpatient Clinic and patients admitted to the Gastroenterology Ward at Alexandria Main University Hospital and the control group was 20 healthy Egyptian subjects with age, sex and BMI matched the CD patients' group.

Crohn's disease was diagnosed based on clinical, radiological, endoscopic and histological examinations. The activity of the CDs was determined according to a combination of clinical and endoscopy indices (the Mayo Clinic index and the Disease Activity Index).

The patients who did not meet the following criteria were excluded; stomach cancer; recent large- or small-intestine surgery within the previous six months; infectious diarrhoea, including parasitic, bacterial and viral diarrhoea; prolonged use of antibiotics in the past; misuse of nonsteroidal anti-inflammatory drug abuse; use of corticosteroids for the previous three months; additional autoimmune diseases; pregnancy, serious burns, sepsis, long-term liver and kidney illnesses, or mental health issues that prevent from giving consent.

Ethical considerations

The study follows the principles of the Declaration of Helsinki (1964) and was approved by the Medical Research Ethics Committee of Alexandria Faculty of Medicine, Egypt with serial number (0305921). All patients provided signed informed consent forms indicating their agreement to take part in the trial and approval for the results to be published.

Clinical Examination

All patients and controls provided a complete history and underwent a full clinical examination.

Investigations

Colonoscopy for all patient groups and controls

Microbiome Study:

Sample collection, preservation and transport

Fresh stool samples were collected from cases and controls and stored in aliquots at −80°C for further processing.

DNA Extraction

Using a QIAamp® Fast DNA Stool Mini Kit (Qiagen, Germany) and following the manufacturer's recommendations, 180–220 mg of stool samples were used to extract DNA. DNA extracts were stored at -80°C until PCR analysis. Two microliters of extracted DNA was subjected to PCR.

SYBR GREEN REAL-TIME PCR

The real-time PCR protocol was performed as described by Tomova et al.[14] Using particular PCR primers, specific phyla, genera, or species that constitute the gut microbiota were targeted. *(Bacteroides, Firmicutes, Ruminococcus, Prevotella, Bacteroidetes, Bifidobacterium, Lactobacillus, C. difficile, A. mucinophilia and F. prausnitzii)*. In addition to the use of a broad-range primer targeting the conserved 16SrRNA sequence of total bacteria, amplification of this sequence served as the denominator against which the amplification of other bacteria was estimated. The primers used in the present study (Metabion International AG, Germany) were previously described[15-20] and are listed in table (1). Amplification was performed in a real-time PCR cycler (Rotor-Gene Q; (Qiagen, Germany) using a SensiFASTTM SYBR® No-ROX PCR Kit (Bioline Co., UK). The reaction mixture was made in a 20 µl volume containing 4 picomoles of each primer.The primers were used as in table (1). The reaction consisted of initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds.[14]

Table 1: Primers used in this study

Target	Primer Name	Primer Sequence (5'-3')
Total bacteria	UnivF UnivR	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTATCCTGTT
A. Muciniphila	AM1-F AM2-R	CAG CAC GTG AAG GTG GGG AC CCT TGC GGT TGG CTT CAG AT
Bacteroides	B3F B3R	CGATGGATAGGGGTTCTGAGAGGA GCTGGCACGGAGTTAGCCGA
Bacteroidetes	Bact934F Bact1060R	GGARCATGTGGTTTATTCGATGAT AGCTGACGACAACCATGCAG
Bifidobacterium	Bif-F Bif-R	TCGCGTC(C/T)GGTGTGAAAG CCACATCCAGC(A/G)TCCAC
Clostridium difficile	C.diff F C.diff R	TTGAGCGATTTACTT CGGTAAAGA TGTACTGGCTCACCTTTGATATTCA
F. Prausnitzii	FPR-2F Fprau645R	GGAGGAAGAAGGTCTTCGG AATTCCGCCTACCTCTGCACT
Firmicutes	Firm934F Firm1060R	GGAGYATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC
Lactobacilli	Lacto-F Lacto-R	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG
Prevotella	PrevF PrevR	CACCAAGGCGACGATCA GGATAACGCCYGGACCT
Ruminococcus	Rflbr730F Clep866mR	GGCGGCYTRCTGGGCTTT CCAGGTGGATWACTTATTGTGTTAA

Statistical analysis:

The data were analysed using the IBM SPSS software package version 20.0. Qualitative data are presented as numbers and percentages. The Kolmogorov–Smirnov test was used to verify the normality of the distribution. Quantitative data are presented as the range (minimum and maximum), mean, standard deviation, median and interquartile range (IQR). The significance of the obtained results was judged at the 5% level.

RESULTS

Demographic Data:

In this study, 24 CD patients 15 males and 9 females were included; their ages ranged from 20 to 44 years old, with a mean age of 28.04 ± 8.46 years. The male to female ratio was 1.66:1. Twenty individuals, with ages ranging from 20 to 42 years and a mean age of 29.75 ± 5.17 years, made up the control group; 9 females and 11 males.

Clinical and Laboratory Data:

There was no positive family history of CD in any of the patients. The illness persisted for an average of one year, ranging from 0.25 to two years. With a mean of 9.05 ± 2.38, the total Mayo score varied from 3 to 11, with 11 (45.8%) having severe Mayo syndrome and 13 (54.2%) having mild Mayo syndrome. The endoscopic scores had a mean ± SD of 6.5 ± 2.15 and ranged from 3 to 11. Four patients (16.7%) had mild CD severity, three patients (12.5%) had moderate CD severity, and seventeen patients (70.8%) had remission. Table 2 shows the different Mayo scores.

Table 2: Distribution of the CD patients according to clinical data

Clinical data	No. (%)
Endoscopic activity	
Mild	13 (54.2%)
Moderate	11 (45.8%)
Endoscopic score	
Mean ± SD.	6.5 ± 2.15
Median (Min. – Max.)	6 (3 – 11)
CD severity	
Mild	4 (16.7%)
Moderate	3 (12.5%)
Remission	17 (70.8%)
Presence of narrowing	
0	17 (70.8%)
1	2 (8.3%)
2	4 (16.7%)
3	1 (4.2%)
Affected surface	
1	3 (12.5%)
2	12 (50%)
3	9 (37.5%)
Ulcerated surface	
1	2 (8.3%)
2	11 (45.8%)
3	11 (45.8%)
Size of ulcers	
1	16 (66.7%)
2	7 (29.2%)
3	1 (4.2%)

Gut microbiome analysis

The quantification of the DNA of certain bacteria was represented relative to the total amount of bacterial DNA found in the faecal sample rather than as an absolute number. The relative abundance values for the various bacteria was 4.75×10^{-5}.

Phylum level analysis

Bacteroidetes turned more prevalent in CD patients., although not statistically significantly more than that of the control healthy group, according to bacterial phylum analysis. On the other hand, Firmicute abundance declined in CD patients, statistically significant (P=0.001). Compared to the control group, CD patients exhibited a significantly decreased Firmicutes/Bacteroidetes (F/B) ratio (2.93, 0.49 respectively) (P=0.003).

Genus level analysis

When compared to the control group, patients with CD had a statistically significant reduction in Ruminococcus (P=0.016). Moreover, comparing the amount of *Bacteroides* or *Prevotella* in the CD patients to the control patients revealed no statistically significant differences (table 3& figure 1). In addition, there was no a significant difference between the CD group and the control group in the Prevotella/Bacteroides ratio (P/B).

Table 3: Comparison between CD patients and healthy control groups according to the gut microbiome

Gut microbiome	CD patients (n = 24)	Healthy control (n = 20)	U	p
F. prausnitzii				
Mean ± SD.	6.06E-2 ± 6.50E-2	2.68E-1 ± 2.10E-1	62.0*	<0.001*
Median (Min. – Max.)	5.08E-2 (2.69E-5 – 2.75E-1)	2.43E-1 (4.0E-3 – 8.66E-1)		
A muciniphila				
Mean ± SD.	9.14E-3 ± 2.66E-2	2.09E-2 ± 6.0E-2	142.0*	0.021*
Median (Min. – Max.)	1.56E-4 (5.62E-7 – 9.47E-2)	1.43E-3 (2.04E-5 – 2.64E-1)		
Ruminococcus				
Mean ± SD.	1.32E-2 ± 1.89E-2	4.55E-2 ± 5.54E-2	138.0*	0.016*
Median (Min. – Max.)	4.66E-3 (4.45E-6 – 6.74E-2)	3.28E-2 (3.21E-4 – 2.14E-1)		
Prevotella				
Mean ± SD.	1.51E-1 ± 1.79E-1	1.59E-1 ± 2.77E-1	230.0	0.814
Median (Min. – Max.)	6.84E-2 (1.99E-4 – 5.15E-1)	1.28E-2 (1.42E-4 – 8.24E-1)		
Lactobacilli				
Mean ± SD.	5.03E-3 ± 6.36E-3	1.36E-1 ± 1.87E-1	68.0*	<0.001*
Median (Min. – Max.)	2.29E-3 (3.55E-6 – 2.08E-2)	7.05E-2 (5.99E-4 – 7.00E-1)		
Firmicutes				
Mean ± SD.	2.64E-1 ± 1.94E-1	5.32E-1 ± 2.59E-1	104.0*	0.001*
Median (Min. – Max.)	1.91E-1 (5.65E-3 – 6.13E-1)	4.63E-1 (8.38E-2 – 9.57E-1)		
C. difficile				
Mean ± SD.	1.23E-3 ± 6.02E-3	0.0E+0 ± 0.0E+0	190.0*	0.032*
Median (Min. – Max.)	0.0E+0 (0.0E+0 – 2.95E-2)	0.0E+0 (0.0E+0 – 0.0E+0)		
Bifidobacterium				
Mean ± SD.	1.51E-2 ± 2.70E-2	1.82E-1 ± 2.74E-1	101.0*	0.001*
Median (Min. – Max.)	5.80E-3 (7.14E-5 – 1.12E-1)	3.56E-2 (1.06E-3 – 8.23E-1)		
Bacteroidetes				
Mean ± SD.	4.77E-1 ± 3.14E-1	3.08E-1 ± 2.83E-1	174.0	0.120
Median (Min. – Max.)	5.16E-1 (1.32E-3 – 9.72E-1)	2.51E-1 (2.15E-3 – 8.72E-1)		
Bacteroides				
Mean ± SD.	2.55E-1 ± 1.70E-1	2.03E-1 ± 1.74E-1	196.0	0.300
Median (Min. – Max.)	2.41E-1 (2.06E-3 – 5.87E-1)	1.39E-1 (1.33E-3 – 5.46E-1)		
P/B				
Mean ± SD.	0.88 ± 1.43	0.99 ± 1.35	230.0	0.814
Median (Min. – Max.)	0.36 (0.0005 – 6.56)	0.24 (0.0003 – 4.31)		
F/B				
Mean ± SD.	13.33 ± 38.55	9.28 ± 15.95	114.0*	0.003*
Median (Min. – Max.)	0.49 (0.03 – 173.83)	2.93 (0.26 – 60.24)		

P/B: Prevotella/Bacteroides ratio
F/B: Firmicutes/Bacteroidetes ratio

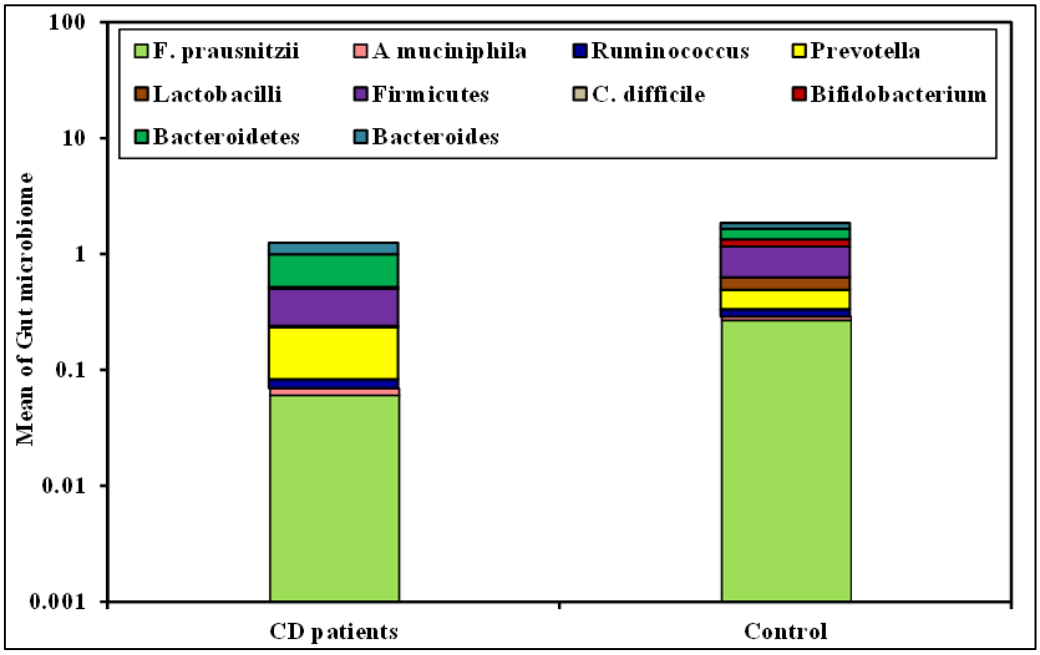

Fig. 1: Comparison between the CD patients and healthy control groups according to Gut microbiome

Species level analysis:

The CD patients exhibited significantly decreased levels of *F. prausnitzii*, *Lactobacilli* (P≤ 0.001), and *Bifidobacteria* (P=0.001) in comparison to those in the control group. Also, When comparing the CD patients to the control group, there was a statistically significant increase in the abundance of *Akkermansia muciniphila* (P=0.021).

With a mean of 1.23E-3 ± 6.02E-3, five individuals with CD tested positive for the pathogenic bacterium *C. difficile*, but none of the control patients did.

Alpha diversity:

There was less microbial diversity in the CD patients than in the healthy controls, according to the Shannon diversity index, which takes into account both species richness and evenness. For CD patients, the diversity indexes' median was 1.34.

Similarity indices:

To evaluate the similarities and dissimilarity between the CD patients and the Healthy Control, the Bray-Curtis similarity index was computed. The mean dissimilarity (difference) between the CD patients and the healthy controls was 44%, with a range of 21-93%.

DISCUSSION

A conflict between intestinal microbiota and mucosal immune system results in intestinal inflammation, which causes Crohn's disease. Research examining the gut microbiome of individuals with IBD has demonstrated that dysbiosis characterized by a relative overabundance of proinflammatory bacteria and a drop of anti-inflammatory bacteria, is a major factor in the etiology of Crohn's disease.[21]

The abundance of *Firmicutes* phylum and *F. prausnitzii* species has dropped considerably in our CD patients, which are the primary beneficial butyrate producers with anti-inflammatory and immune-modulatory roles in gut homeostasis through the induction of mucin formation. Our findings were consistent with Fujimoto et al,[22] Sokol et al.,[23] demonstrating that CD patients had dysbiosis and much lower abundances of the phyla *Firmicutes* and *F. prausnitzii* than did healthy individuals. Our data support the findings of numerous other studies that reported a decreased relative abundance of *F. prausnitzii* in CD patients. These studies also demonstrated that this genus is not missing in UC patients, making it a viable marker to distinguish between CD and UC patients.[24]

Additionally, CD patients exhibited much lower levels of *Bifidobacteria* (P=0.001) and *Lactobacilli* (P≤ 0.001) than healthy controls in the current investigation. These helpful bacteria aid in the production of mucus and the decrease of inflammation. These results agrees with Morgan et al. [25] and Gevers et al., [26] who revealed that IBD patients had lower levels of *Lactobacillus* and *Bifidobacterium*.

In the CD patients, the Firmicutes/Bacteroidetes (F/B) ratio was decreased (0.49) than in the control group (2.93); there is a significant difference between these two ratios (P=0.003). This finding agrees with Pascal et al [27] who has demonstrated that decreased F/B ratio may act as a biomarker of gut dysbiosis in CD patients.

In the current investigation, patients with CD exhibited a significantly decreased *Ruminococcus* abundance (P=0.016) than those in the control group. This result was consistent with the findings of Morgan et al[25] and Frank et al [28] who demonstrated that IBD patients had lower Ruminococcus abundance, especially for a species that produces butyrate. Nevertheless, there were no statistically significant variations in the P/B ratio or the abundance of Bacteroides or *Prevotella*. However, examination of the gut microbiota in individuals with CD revealed a favorable relationship between the degree of the illness and the amount of H2S-producing bacteria like *Prevotella*.[29]

Five CD patients (20%) tested positive for the pathogenic bacteria *Clostridium difficile* in the current investigation, with a mean value of 1.23E-3 ± 6.02E-3.. This result is in line with recent studies that discovered 20% of IBD patients had *Clostridium difficile* infection.[30] This could be a finding of IBD exacerbation or experience higher rates of recurrence.[31]

It has been demonstrated that there are substantial differences in the diversity and load of the gut microbiomes between healthy individuals and those with IBD.[32] The Shannon index revealed that the alpha diversity of our research groups was comparable. A predicted outcome considering that the study's scope was limited to certain bacterial genera and/or species.

Gut microbial diversity has previously been determined as a unique biomarker of healthy metabolic capacity, besides its ability to protect the human the gastrointestinal tract from external stresses.[33] Therefore, when evaluating patients with non-specific signs and symptoms indicative of IBD, a non-invasive diagnostic tool like the one reported here may be helpful in making clinical decisions when the diagnosis of CD is initially unclear.

CONCLUSION

When making a judgment in cases of CD, this microbial signature may be helpful when the diagnosis is first unclear. Although this study makes accurate and useful use of prior knowledge about microbial genomes, the data it provides only reflects one stage in the functional investigation of the IBD microbiota. To further define the consequences of medication usage and the host's altered microbiota linked to IBD, more research is required focusing on proteomic or metabolomics characterization and dietary meta-data.

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