

LEADING ARTICLE

# The relationship between the commensal microbiota levels and Crohn's disease activity

Hagamenon de Alencar Junior,\* Ana Paula Ribeiro Paiotti,<sup>†</sup> Humberto Bezerra de Araújo Filho,<sup>‡</sup> Celina Tizuko Fujiyama Oshima,\* Sender Jankiel Miszputen<sup>†</sup> and Orlando Ambrogini-Júnior<sup>†</sup>

\*Department of Pathology, <sup>†</sup>Division of Gastroenterology and <sup>‡</sup>Division of Pediatric Gastroenterology, Universidade Federal de São Paulo—Paulista Medical School, UNIFESP, São Paulo, Brazil

**Key words**

bacilli, bacteroidetes, bifidobacteriaceae and enterobacteriaceae, Crohn's disease, gut microbiota.

Accepted for publication 28 March 2020.

**Correspondence**

Ana Paula Ribeiro Paiotti, Laboratory of Hepatology Molecular Applied, Division of Gastroenterology, UNIFESP/EPM. Rua Pedro de Toledo, 669, 5th floor, São Paulo, 04039-032 Brazil.  
Email: a.paiotti@unifesp.br

**Declaration of conflict of interest:** This work was performed at São Paulo Hospital, Universidade Federal de São Paulo, Paulista Medical School, UNIFESP/EPM, São Paulo, SP, Brazil. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES), Finance Code 001. All authors designed the study, interpreted the data, and wrote the manuscript. The authors declare no conflict of interest.

**Funding support:** Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) Finance Code 001

## Introduction

Crohn's disease (CD) is a chronic granulomatous inflammatory disorder that may affect any portion of the gastrointestinal tract (GI tract).<sup>1,2</sup> While the precise etiology remains unclear, several studies suggest that intestinal inflammation arises due to an abnormal response of the intestinal immune system to the fecal microbiota in genetically predisposed individuals.<sup>3-7</sup>

The human gut harbors more than 10<sup>14</sup> microorganisms, comprising more than 500–1000 species,<sup>8-10</sup> including bacterial, microeukaryotic, and viral populations,<sup>11</sup> which form a unique ecosystem allowing a symbiotic relationship. This relationship contributes to both health and disease status of the host.<sup>12</sup> The gut microbiota plays an important role in inhibiting the growth and/or

## Abstract

**Background and aim:** Human gut microbiota play an important role in metabolism and host physiology. Perturbations of the gut microbial communities lead to the development of various diseases such as inflammatory bowel disease, celiac disease, allergic diseases, and metabolic diseases. Crohn's disease is a chronic inflammatory bowel disease characterized by periods of remission and relapse. Several studies suggest that intestinal inflammation arises due to an abnormal response of the intestinal immune system to the fecal microbiota. The goal of the study was to evaluate the relative amount of four bacterial groups in fecal samples of Crohn's disease patients and their relation to the inflammatory activity.

**Methods:** We studied stool samples of 105 individuals, 54 with Crohn's disease and 51 as a control group. The DNA extracted from the stool samples was subjected to real-time polymerase chain reaction (qPCR) for quantification of the Bacteroidetes phylum, class Bacilli, and Bifidobacteriaceae and Enterobacteriaceae families.

**Results:** We found a significant increase in Bacteroidetes in Crohn's disease samples when compared to the control group (14 650 and 2060 CFU/ng DNA, respectively) ( $P = 0.014$ ). On the other hand, we observed a significant reduction in Bacilli and Bifidobacteriaceae (13 and 58 CFU/ng DNA, respectively) ( $P < 0.0001$ ). In contrast, patients without any drug treatment presented an increase of Bacilli and Bifidobacteriaceae (102 521 and 6235 CFU/ng DNA, respectively) ( $P < 0.0001$ ).

**Conclusion:** The commensal bacteria were decreased in fecal samples of participants with Crohn's disease when compared to the control group. There was no relation between the disease location and/or disease activity with the microbiota.

colonization of incoming pathogens by competing for limited nutrients, mucus production and enhancement of epithelial cell barrier function, the release of antimicrobial peptides, and the production of immunoglobulins.<sup>13-15</sup> The metabolites generated by the gut microbiota are essential for the maintenance of intestinal homeostasis.<sup>16</sup> Short-chain fatty acids (SCFAs), such as acetate and butyrate formed by the anaerobic bacteria fermentation of dietary fiber, regulate anti-inflammatory immune responses and enhance epithelial cell barrier function.<sup>16,17</sup> In this way, the gut microbiota are strongly associated with host physiology, and perturbations of the gut microbial communities (dysbiosis) lead to the development of various GI tract disorders, such as CD.<sup>18,19</sup>

Patients with CD showed a reduction of microbial diversity evidenced by the increase in Bacteroidetes and Enterobacteriaceae

and a decrease in Firmicutes species.<sup>20</sup> The lower proportion of *Faecalibacterium prausnitzii*, a member of the phylum Firmicutes with anti-inflammatory properties, increased the risk of postoperative recurrence after resection for the ileal disease.<sup>21</sup> Other butyrate-producing bacterial species, such as *Blautia faecis*, *Roseburia inulinivorans*, *Ruminococcus torques*, *Clostridium lavalense*, and *Bacteroides uniformis*, were also shown to be significantly reduced in CD patients.<sup>22</sup> Based on this evidence, we evaluated the relative amount of four bacterial groups (Bacteroidetes phylum, class Bacilli, Bifidobacteriaceae family, and Enterobacteriaceae family) in the fecal samples of CD patients and their relation to the inflammatory activity.

## Materials and methods

**Participant's collection data.** In a convenience sample, a cross-sectional study included 105 participants, 54 with CD and 51 without GI tract disease (defined as a healthy control group). All the participants with CD were followed up in the Inflammatory Bowel Disease Department of São Paulo Hospital, Universidade Federal de São Paulo, Paulista Medical School, Brazil (UNIFESP/EPM). The participants of the control group were recruited from the Central Laboratory of São Paulo Hospital, UNIFESP/EPM.

Sociodemographic data, such as age, gender, ethnicity, and type of birth (vaginal or cesarean section), were collected from all participants. In addition, medical records of patients with CD, such as diagnostic time, disease evolution, disease phenotype, disease location, treatment, and clinical activity classification according to the Harvey-Bradshaw index (HBI),<sup>23</sup> were used. Participants who used antibiotics in the last 3 months were excluded from this study, which was carried out from November 2015 to May 2016.

**Ethical considerations.** Written informed consent was obtained from each participant, and the study protocol was approved by the Ethics Committee of the Universidade Federal de São Paulo, UNIFESP (Number 1199/2015). The study protocol was in accordance with the ethical principles for medical research involving human subjects of the Helsinki Declaration.

### Fecal microbiota analysis

**Stool collection and DNA extraction.** Fresh stool samples were collected from each study participant. The sample from each participant was homogenized and divided into three aliquots

and immediately stored at  $-80^{\circ}\text{C}$  for DNA extraction. The bacterial genomic DNA was extracted from the stool sample using the QIAmp Qiagen mini stool kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purified DNA was diluted to a final volume of 200  $\mu\text{L}$ . The DNA concentration was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). All the DNA samples were diluted to a final concentration of 20  $\text{ng}/\mu\text{L}$  and stored at  $-20^{\circ}\text{C}$ .

**Quantitative real-time PCR.** The quantitative real-time polymerase chain reaction (qPCR) was performed on a Rotor-gene Q thermocycler (Qiagen, Hilden, Germany). SYBR Green PCR Master Mix (Thermo Fisher Scientific) was used to amplify the gene of a specific bacterial group. The primers used to amplify the specific bacterial groups are listed in Table 1.<sup>24</sup> Each PCR was carried out in duplicate with a final volume of 10  $\mu\text{L}$ , comprising of 5.0  $\mu\text{L}$  SYBR Green PCR Master Mix (Thermo Fisher Scientific), 0.2  $\mu\text{L}$  each of the primers (10  $\text{pmol}/\mu\text{L}$ ), 0.5  $\mu\text{L}$  of the DNA sample, and 4.1  $\mu\text{L}$  of DEPC-treated water (Qiagen).

Thermocycling was performed under the following conditions:  $95^{\circ}\text{C}$  for 5 min followed by 40 cycles of  $95^{\circ}\text{C}$  for 10 s and  $60^{\circ}\text{C}$  for 15 s, a dissociation cycle for the melting curve of  $95^{\circ}\text{C}$  for 1 min, and a melting curve program of  $70\text{--}95^{\circ}\text{C}$  with a gradual temperature increase of  $1^{\circ}\text{C}/\text{s}$ . As a negative control, a reaction using all the reagents except the DNA sample was included, and its specificity was confirmed by sequencing and alignment using the BLAST system.

The standard curve for all the analyses was created by amplifying a TopoTA plasmid (Invitrogen<sup>TM</sup>) carrying a fragment of the reference gene previously amplified by conventional PCR. With the molecular mass of the plasmid and insert known, it is possible to calculate the copy number as follows: mass in Daltons ( $\text{g}/\text{mol}$ ) = (size of double-stranded [ds] product in base pairs [bp]) ( $330 \text{ Da} \times 2 \text{ nucleotides [nt]/bp}$ ).<sup>25</sup> Hence, the  $\text{g}/\text{mol}$  value divided by Avogadro's number equals the  $\text{g}/\text{molecule}$  value, which equals the copy number.<sup>25</sup> Knowing the copy number and concentration of plasmid DNA, the precise number of molecules added to subsequent real-time PCR runs can be calculated, thus providing a standard for the specific copy number of gene quantification. The real-time PCR results were expressed as colony-forming units/g of feces (CFU/g of feces) once *M. smithii* possessed one copy of the nifH gene per cell.<sup>26</sup>

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism (version 7.0) and STATA (version 14).

**Table 1** Sequences used to amplify the specific bacterial groups<sup>24</sup>

Bacteria	Direction	Sequence
Bacteroidetes phylum	Forward	5'CGGAWTYATTGGGTTTAAAGGG3'
	Reverse	5'GGTAAGGTTCTCGCGTA3'
Class Bacilli (Firmicutes phylum)	Forward	5'GCAGTAGGGAATCTTCCGC3'
	Reverse	5'ACACTTAGCACTCATCGTTT3'
Bifidobacteriaceae family (Actinobacteria phylum)	Forward	5'CTCCTGGAAACGGGTGG3'
	Reverse	5'CTTTCACACCRGACGCG3'
Enterobacteriaceae family (Proteobacteria phylum)	Forward	5'CGTCGCAAGMMCAAAGAG3'
	Reverse	5'TTACCGGGCTGCTGGCAC3'

**Table 2** Sociodemographic characteristics of all participants

		Crohn's disease group, n (%)	Control group, n (%)	P value
Age	Mean $\pm$ SD	44.03 $\pm$ 16.87	48.56 $\pm$ 14.35	0.1197
	Min./max.	17/84	13/77	
Gender	Women	22 (40.74)	41 (80.39)	0.0003
	Men	32 (59.25)	10 (19.60)	
Ethnicity	White	42 (77.77)	37 (72.54)	0.5294
	Black	12 (22.22)	13 (25.49)	
	Asian	—	1 (1.96)	
Type of birth	Vaginal	40 (74.07)	45 (88.23)	0.0646
	Cesarean	14 (25.92)	5 (9.80)	
	Adopted	—	1 (1.96)	

Values are expressed as means  $\pm$  SD. Chi-square test and *t*-test.

Error bars graphs were used. A value of  $P < 0.05$  was considered statistically significant.

Data were reported as frequency (%) for the descriptive analysis of participants' characteristics and median and quartiles for bacterial relative quantification. Chi-square test and *t*-test were used for sociodemographic data. Mann-Whitney test was used for fecal microbiota quantification. Comparison between fecal microbiota and clinical activity and/or disease location in participants with CD was performed using ANOVA (Kruskal-Wallis).

## Results

### Sociodemographic aspects of all participants.

Sociodemographic characteristics of all participants are shown in Table 2. Of the 54 participants with CD, 32 (59.25%) were men, and 22 (40.74%) were women, with a mean age of 44.03 years. In the healthy control group ( $N = 51$ ), the mean age was 48.56 years, with most being women (80.39%).

**Table 3** Clinical characteristics of Crohn's disease group

		n (%)
Diagnostic time	0 month to 10 years	25 (46.29)
	11–20 years	18 (33.33)
	21–30 years	6 (11.11)
	Up 30 years	5 (9.25)
Disease phenotype	Obstructive	19 (35.18)
	Fistulizing	6 (11.11)
	Inflammatory	9 (16.66)
	Associated phenotype	20 (37.03)
Disease location	Small bowel affected	43 (79.63)
	Large bowel affected	11 (20.37)
Treatment	Without treatment	2 (3.70)
	Only anti-TNF	7 (12.96)
	Without anti-TNF	14 (25.92)
	Combination therapy with anti-TNF	20 (37.0)
	Combination therapy without anti-TNF	7 (12.96)
	Lost data	4 (7.40)
Harvey-Bradshaw index	Activity (score $\geq$ 5)	30 (55.60)
	Remission (score $<$ 5)	24 (44.44)

Most participants declared their ethnicity to be white (77.77% of the CD group and 72.54% of the healthy control group). The type of birth was also studied, and the majority was vaginal. One participant declared to be adopted.

### Clinical characteristics of participants with CD.

According to the diagnostic time, 25 of 54 participants (46.29%) presented up to 10 years of disease evolution. Concerning the clinical features, 19 of 54 (35.18%) presented obstructive disease, and 20 of 54 participants (37.03%) presented more than one feature at the same time (fistulizing, obstructive, and inflammatory), described as an associated phenotype in Table 3. In relation to the disease location, 43 of 54 participants (79.62%) presented ileal involvement.

In relation to the treatment, 27 of 54 participants (49.96%) received combination therapy [20 (37%) with anti-TNF and 7 (12.96%) without anti-TNF]. About 14 of 54 (25.92%) patients were making use of a single treatment with anti-inflammatory medications, corticosteroids, or immunosuppressors, and 7 of 54 (12.96%) received only anti-TNF. Only two patients were untreated, and the other four were lost data.

According to the clinical evaluation by HBI, 55.6% presented activity disease (HBI  $\geq$  5) and 44.44% clinical remission. The clinical characteristics are summarized in Table 3.

**Quantification of fecal microbiota by qPCR.** Our results showed an increase of Bacteroidetes in samples of the CD group when compared to the healthy control group ( $P = 0.0140$ ). On the other hand, we observed a significant decrease of Bacilli and Bifidobacteriaceae ( $P < 0.0001$ ). Regarding Enterobacteriaceae, we

**Table 4** Quantification of fecal microbiota by qPCR

Bacteria (CFU/ng DNA)	Control (n = 51) median (25–75%)	Crohn's disease (n = 54) median (25–75%)
Bacteroidetes	2060 (346–7000)	14650** (306.5–96 100)
Bacilli	2100 (1130–4480)	13* (6–89.25)
Bifidobacteriaceae	3830 (2140–5300)	58* (6–343)
Enterobacteriaceae	111 (43–1220)	609 (62.5–2768)

Values are expressed as medians  $\pm$  interquartile (25–75%).

\* $P < 0.0001$  Mann-Whitney test;

\*\* $P = 0.014$  Mann-Whitney test.

**Table 5** Comparison between fecal microbiota and clinical activity and disease location in participants with Crohn's disease (CD)

	Bacteroidetes Median (25–75%)	Bacilli Median (25–75%)	Bifidobacteriaceae Median (25–75%)	Enterobacteriaceae Median (25–75%)
Activity ( <i>n</i> = 30)	13 150 (279.5–59 050)	11.5 (5.75–89.25)	59 (5.5–245.50)	242 (46.5–3003)
Remission ( <i>n</i> = 24)	16 600 (443.3–129 675)	17 (6–134.5)	53.5 (7–470.5)	1320 (225.5–2700)
Disease location				
SB affected ( <i>n</i> = 43)	11 700 (299–37 333)	13 (6–124)	60 (6–136)	717 (94–3185)
LB affected ( <i>n</i> = 11)	52 300 (9330–222 000)	16 (6–82)	27 (2–343)	180 (34–1930)

Values are expressed as medians ± interquartile (25–75%). ANOVA (Kruskal–Wallis), *P* > 0,05 (nonsignificant). LB, large bowel; SB, small bowel.

**Table 6** Comparison between fecal microbiota and type of treatment in participants with Crohn's disease (CD)

Type of treatment	Bacteroidetes Median (25–75%)	Bacilli Median (25–75%)	Bifidobacteriaceae Median (25–75%)	Enterobacteriaceae Median (25–75%)
Without treatment ( <i>n</i> = 2)	124 334 (1.0–248 666)	102521* (42.00–205 000)	6235* (4.000–12 466)	2864 (1028–4700)
Only anti-TNF ( <i>n</i> = 7)	192.0 (74.00–18 000)	9.000 (6.000–13 416)	3.000 (1.000–64.00)	33.00 (19.00–10 242)
Without anti-TNF ( <i>n</i> = 14)	79 833 (8843–180 916)	11.00 (4.750–20.25)	15.00 (5.500–152.3)	401.5 (112.0–2114)
Combination therapy with anti-TNF ( <i>n</i> = 20)	9017 (505.5–29 258)	16.00 (7.250–206.3)	78.50 (38.00–483.3)	1119 (78.25–3371)
Combination therapy without anti-TNF ( <i>n</i> = 7)	22 733 (610.0–140 666)	16.00 (5.000–29.00)	56.00 (11.00–376.0)	1457 (717.0–2000)

Values are expressed as medians ± interquartile (25–75%).

\**P* < 0.0001 ANOVA (Kruskal–Wallis).

did not observe differences. The results are reported in Table 4 and in Figures S1–S4.

### Comparison between fecal microbiota, type of treatment, clinical activity, and disease location in participants with CD.

The comparison between fecal microbiota and clinical activity did not show significant differences. In the same way, there were no significant differences regarding the disease location. In contrast, the comparison between the microbiota and type of treatment demonstrates a significant difference. Patients without any drug treatment presented an increase of Bacilli and Bifidobacteriaceae (*P* < 0.0001). However, because of the small sample size, this result should be interpreted with caution. The results are summarized in Tables 5 and 6.

## Discussion

The exact mechanisms of the association between the compositional and metabolic change of the intestinal microbiota in inflammatory bowel disease (IBD) pathogenesis are unclear. Several studies are conducted with different methodologies and sample types but face difficulty in data comparison.

Except for the 16S rRNA sequencing, which supplies more robust data, the qPCR methodology of some bacterial groups can be used preliminarily in the understanding of the intestinal microbiota. In this way, we evaluated the relative amount of four bacterial groups (Bacteroidetes phylum, class Bacilli, Bifidobacteriaceae family, and Enterobacteriaceae family) in the samples of CD patients and their relation to inflammatory activity.

Our findings demonstrate an increase of Bacteroidetes in fecal samples of participants with CD in comparison with a healthy

control group. However, we did not find any relation between this phylum and the clinical activity or disease location. Similar results were found in the gut biopsy and blood of patients with CD in both conditions (activity and remission).<sup>27</sup> In contrast, another study observed depletion of Bacteroidetes in ileal biopsy.<sup>28</sup> A study with 190 samples of biopsy of CD patients showed a decrease in Bacteroidetes, suggesting that the microbial communities of the distal small gut wall are similar to the large gut, but they differ in the relative proportion of subgroups present.<sup>29</sup>

Bacteroidetes are anaerobic Gram-negative bacteria that play an important role in a metabolic activity, such as polysaccharide production regulating the balance of pathogenic agents and regulatory T cells,<sup>30</sup> as well as in carbohydrates metabolism, nutrition, and health maintenance.<sup>31</sup> In addition, they are responsible for the secretion of interleukin-10 (IL-10), which in turn suppresses the production of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 17 (IL-17), and interleukin 23 (IL-23).<sup>30</sup>

Our findings also demonstrated a significant decrease in levels of Bacilli (Firmicutes phylum) in CD patients. However, we cannot state which gender or species within that class influenced these results. The presence of *Lactobacillus stirpes* is fundamental in epithelial barrier fortification through several mechanisms such as mucin secretion, better functioning of tight junctions, positive regulation of heat shock proteins, and apoptosis prevention of epithelial cells.<sup>32</sup>

An interesting study showed alterations in Firmicutes and Bacteroidetes communities in the gut biopsy of IBD patients. Both inflamed and noninflamed tissue demonstrate a decrease in Firmicutes and an increase in Bacteroidetes in CD patients.<sup>20</sup> Regarding the Bifidobacteriaceae levels, we observed a significant decrease also in CD patients. This group of bacteria is very

important in gut defense because of the induction of anti-inflammatory mediators.<sup>33,34</sup>

Lactobacillus and Bifidobacterium are lactic acid bacteria that can produce lactic and acetic acid, respectively, which may lower intestinal pH and suppress the growth of various pathogenic bacteria. These probiotic bacteria may also produce various substances such as hydrogen peroxide, organic acids, bacteriocins, and biosurfactants, which are toxic to pathogenic bacteria.<sup>35,36</sup>

In the pathogenic bacteria group, the adherent invasive *Escherichia coli* and *Salmonella* sp., both included in the Proteobacteria phylum, are the common types of bacteria in IBD patients, which can exacerbate the inflammation. This probably occurs through the production of hydrogen sulphate and other toxic subproducts, as well as the decrease in the availability of beneficial metabolites such as butyrate.<sup>37</sup> Butyrate is effective in decreasing TNF- $\alpha$  and other proinflammatory cytokines in the gut tissue of CD patients, and its reduction is associated with a decrease in Firmicutes.<sup>38</sup> Our data did not demonstrate significant differences in Enterobacteriaceae quantification.

The data around the gut microbiota composition are heterogeneous. The difference between the number of bacteria can be found through the sample type analyzed (tissue or feces), area (inflamed and noninflamed tissue),<sup>20,39,40</sup> age,<sup>41–43</sup> gender,<sup>44,45</sup> and weight.<sup>46</sup> In addition, different levels of the same phylum can be observed along the gut. Despite this, it is still not clear if dysbiosis it is the cause or the consequence of chronic intestinal inflammation.

In summary, our findings demonstrated an important decrease in Bifidobacteriaceae and Bacilli in fecal samples of patients with CD. These data make us question if this could be indicative of relapse or worsening of the clinical condition, although we do not observe significant differences between disease location and/or disease activity and the microbiota.

## Acknowledgments

The authors express their gratitude to all participants of this protocol and to the Division of Pediatric Gastroenterology, Universidade Federal de São Paulo, Paulista Medical School, UNIFESP, SP, Brazil. They also thank Prof. Dr. Mauro Batista de Moraes for assistance in qPCR execution.

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## Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website:

**Figure S1** The concentration of Bacteroidetes in fecal samples of the control group and Crohn's disease group (\**P* = 0.0140 Mann–Whitney test)

**Figure S2** The concentration of Bacilli in fecal samples of the control group and Crohn's disease group (\**P* < 0.0001 Mann–Whitney test)

**Figure S3** The concentration of Bifidobacteriaceae in fecal samples of the control group and Crohn's disease group (\**P* < 0.0001 Mann–Whitney test)

**Figure S4** The concentration of Enterobacteriaceae in fecal samples of the control group and Crohn's disease group (not significant, Mann–Whitney test)