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Review

GUT DYSBIOSIS AND PAEDIATRIC CROHN'S DISEASE

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Running title: Dysbiosis and Crohn's disease.

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HIGHLIGHTS

- Crohn's disease (CD) derives from an imbalance between the host defences and environmental factors.
- Numerous genetic variations in autophagy influence CD risk.

- Gut microbiota are primary actors in Crohn's disease (CD)'s pathogenesis.
- Development and course of CD of children is associated with gut dysbiosis.
- Further studies are needed to discover new therapeutic options for CD.

SUMMARY

Objectives: The main objective of this manuscript is to discuss our present knowledge of the relationships between dysbiosis and paediatric Crohn's disease (CD). The therapeutic role of the methods currently used to re-establish normal gut microbiota composition is also analysed.

Methods: PubMed was used to search for all of the studies published from January 2008 to June 2018 using the key words: "Crohn's disease" and "gut dysbiosis" or "microbiota" or "microbioma" or "probiotic" and "children" or "paediatric". More than 100 articles were found, but only those published in English or providing evidence-based data were included in the evaluation.

Results: Gut microbiota are primary actors in CD's pathogenesis. The new techniques developed in metagenomics allow us to reveal new details of microbiota composition in healthy subjects and CD patients, and to elucidate the link between microbiota and numerous pathologies, such as obesity, allergies and type 1 diabetes mellitus.

Conclusion: Discoveries on the role of gut microbiota could potentially disclose new therapeutic options for CD treatment and improve the existing therapies. Further

47 studies are needed to facilitate the diagnosis and tailor the therapy of a pathology that is
48 an increasing burden on public health.
49
50 Key-words: Crohn's disease; dysbiosis; gut microbiota; inflammatory bowel disease.

INTRODUCTION

Crohn's disease (CD) is a chronic, relapsing inflammatory bowel disease (IBD) that may affect any section of the gastrointestinal tract in a non-contiguous pattern [1]. Global prevalence of the disease is greater in the Western world compared to developing countries, but a sharp increase in new CD cases has been observed in newly industrialized geographic areas in recent years [2]. The prevalence is approximately 0.3% in Europe and North America and approximately 20-40 per 100,000 in Asia and South America [3]. Approximately 25% of the cases are diagnosed in children. Rates of CD increase from the first year of life, and the highest rates appear in adolescence. The prevalence of CD in paediatric patients is increasing significantly worldwide, even in Western nations where global prevalence is relatively stable [3, 4]. Whether immigration from newly industrialized nations or other factors, such as changes in lifestyle, diet, urbanisation and other environmental changes, favour the development of CD in the paediatric population is not known.

CD lesions involve all layers of the bowel wall (i.e., transmural), unlike ulcerative colitis (UC). The paediatric CD phenotype is slightly different than adult CD. Childhood CD is characterized with a panenteric phenotype at onset. Lesions can be detected in the ileocolonic and upper gastrointestinal tracts in 43% of cases in paediatric CD, but these occur in only 3% of adult patients. In contrast, isolated ileal and colonic diseases are significantly less common in paediatric CD (2% vs 31%, $p<0.0001$, and 15% vs 36%, $p<0.0001$, respectively) [5]. The natural history of CD exhibits significant individual variation. Up to 30% of cases exhibit an indolent course without the need for

immunosuppressive drugs or surgery, but greater than 50% require surgery within 2 decades of diagnosis [6, 7]. However, children generally exhibit a more severe course, especially those with a very early onset. CD alters nutrition and growth because of the very severe gastrointestinal manifestation, which compromises normal linear growth and pubertal development [8].

The pathogenesis of CD is not precisely defined, but evidence suggests that CD likely derives from an uncorrected imbalance between the host defences and environmental factors, including the gut microbiota, in genetically susceptible individuals. Numerous genetic variations that influence CD risk have been identified [9]. *NOD2, IL23R, ATG16 L1, IRGM, IL10, NKX2-3* and *ORMDL3* gene modifications are those most frequently found in CD patients [10]. These genes are involved in innate immunity and primarily autophagy, and their mutations significantly alter the immune system response to bacteria in the digestive tract [10]. This altered response likely leads to persistent bacterial infections, a defective mucosal barrier and an imbalance in the regulation of the intestinal immune response with loss of tolerance. The role of environmental factors in favouring persistent inflammation is supported by evidence that CD is significantly more common in industrialized geographic areas and, among adults, in smokers. However, the strong modification of gut microbiota composition, i.e. dysbiosis, is the most important environmental factor associated with the development and maintenance of CD. This paper discusses our present knowledge of the relationships between dysbiosis and paediatric CD. The therapeutic role of the methods currently used to re-establish normal gut microbiota composition is also analysed. PubMed was used to search for all of the studies published from January 2008 to June

2018 using the key words: “Crohn’s disease” and “gut dysbiosis” or “microbiota” or “microbioma” or “probiotic” and “children” or “paediatric”. More than 100 articles were found, but only those published in English or providing evidence-based data were included in the evaluation.

GUT MICROBIOTA FUNCTIONS

Microbiota perform several functions that are essential for health (Table 1). Microbiota synthesize vitamins and improve the metabolism of nutrients, such as polysaccharides and polyphenols, via numerous enzymes that are not encoded by the human genome. Saccharolytic bacterial fermentation generally produces beneficial metabolites, such as short-chain fatty acids (SCFAs) [11], which are also produced by peptide and amino acid (glutamate, lysine, histidine, cysteine, serine, and methionine) fermentation [12]. The most important SCFAs are acetate, propionate, and butyrate. Several bacteria produce acetate, but the *Bacteroides* species, *Negativicutes*, and some *Clostridium* species primarily produce propionate. Butyrate production is strictly related to the presence of *Firmicutes*, including some *Lachnospiraceae* and *Faecalibacterium prausnitzii*. Acetate is the most abundant SCFAs, and it regulates the growth of other beneficial bacteria. Some of these bacteria, such as *Faecalibacterium prausnitzii*, cannot grow in the absence of acetate in the culture medium [13]. Acetate enters cholesterol metabolism and regulates appetite [14]. Propionate is converted to glucose during intestinal gluconeogenesis, and reduces the risk of obesity via appetite regulation [15]. Butyrate is the most important energy source for colonic mucosal cells, and it seems essential for the conservation of mucosal integrity. In vitro studies

121 demonstrated that butyrate decreased pro-inflammatory cytokine expression via the
122 inhibition of lipopolysaccharide-induced nuclear factor kappa B activity, which is
123 involved in the transcription of these genes [16]. Butyrate favours the production of
124 mucin and antimicrobial peptides and enhances the maintenance of colonic
125 homeostasis via the regulation of fatty acid metabolism, electron transport and oxidative
126 stress pathways [17]. Butyrate also reduces the risk of colon cancer development via
127 the induction of colon cancer cell apoptosis [18].

128 Gut microbiota regulate immune system function. Development and maturation of
129 the gut immune system depend on the presence of gut microbiota. Germ-free animals
130 exhibited a reduced number of intra-epithelial lymphocytes, reduced sizes and numbers
131 of Peyer's patches, altered crypt structure, and fewer goblet cells compared to normal
132 subjects, which lead to a reduced mucous thickness [19, 20]. Immune intestinal
133 homeostasis partially depends on the balance between the effector arm of the immune
134 system, which is led by effector CD4+ T cells, and the regulatory arm, which is led by
135 regulator CD4+ T cells (Treg cells). The effector arm recognizes and eliminates
136 pathogens, and the regulatory arm suppresses inflammation and promotes immune
137 tolerance [21]. Different gut bacteria modulate arm efficiency to favour and reduce
138 inflammation and the risk of CD development. Firmicutes are generally protective
139 because these bacteria stimulate the regulatory arm. For example, the spore-forming
140 *Clostridium* species in clusters XIVa and IV are associated with high concentrations of
141 Treg cells [22], which produce high levels of interleukin (IL)-10 [23]. *Faecalibacterium*
142 *prausnitzii* is the most important of these bacteria, and its presence is essential for
143 normal gut function and health [24]. However, whether all or only some strains offer this

protection is not clear [25]. Other bacteria, such as *Lactobacilli* and *Bifidobacteria*, are proposed to induce Treg cells [26]. In contrast, *Proteobacteria* seem to favour inflammation by bolstering the effective arm of the intestinal immune system. Several studies demonstrated that so-called segmented filamentous bacteria (SFB) in combination with *Helicobacter hepaticus*, several members of *Enterobacteriaceae* and *Bacteroides fragilis* induce Th1 and Th17 responses in the gut [27]. This stimulation enhances resistance to invading bacteria, but it may be very deleterious when chronically present or poorly controlled. Colonization with SFB results in hypersensitivity to colitis in T-cell-dependent models of IBD [28] and increases the development of Th17-mediated arthritis in susceptible mice [29].

GUT MICROBIOTA COMPOSITION IN CHILDREN

Several studies evaluated gut microbiota composition in healthy children and paediatric patients with CD. However, evaluation of these studies is not easy. The most important limiting factor is the different compositions of mucosal and luminal microbiota. Most studies focused on luminal/faecal bacteria instead of the bacterial communities adherent to the intestinal mucosa. These two intestinal compartments possess significantly different microbial communities. Gut content of nutrients is significantly higher in the upper intestinal tract, which plays a relevant role in favouring the type and amount of bacteria that influence gut structure and function. Mucosal microbiota are in closer proximity to immune cells and may exert a stronger influence on the gut immune system [30]. However, present knowledge of the relationships between gut microbiota

166 composition and CD development and maintenance are adequate to draw some
167 conclusions and suggest some reasonable therapeutic approaches.
168
169 **Healthy children**
170 The definitive composition of gut microbiota is not achieved before the end of the
171 third year of life. The relative abundance of the different phyla at birth and during the
172 first months of life can differ markedly between individuals because of the influence of
173 several factors, such as the mother's characteristics (i.e., malnutrition or over-nutrition,
174 obesity, diabetes, eczema, and stress during pregnancy), prematurity, type of delivery,
175 feeding, and antibiotic administration (Table 2). All of these factors significantly alter
176 ideal gut microbiota composition of these subjects and delay the achievement of
177 microbiota characteristics that are generally detected in healthy older children,
178 adolescents and adults [31]. When a mature gut microbiota is established, it is primarily
179 based on four major phyla that cover more than 90% of the total bacterial population
180 (*Firmicutes, Bacteroidetes, Actinobacteria*, and *Proteobacteria*), but several additional
181 minor phyla, such as *Verrucomicrobia* and *Fusobacteria,* can be identified [32]. The
182 *Firmicutes* phylum is composed of Gram-positive aerobic and anaerobic bacteria.
183 Prominent members are included in the genera *Lactobacillus, Enterococcus,*
184 *Clostridium, Ruminococcus, Streptococcus, Staphylococcus, Escherichia,* and
185 *Klebsiella*. *Bacteroidetes* are Gram-negative bacteria and include the genera
186 *Bacteroides* and *Prevotella*. *Actinobacteria* are Gram-positive bacteria, and genera
187 *Bifidobacterium, Corynebacterium, Propionibacterium,* and *Atopobium*, are the most

188 frequently detected. The *Proteobacteria* phylum contains Gram-negative bacteria, most
189 notably the family of *Enterobacteriaceae*, including *Enterobacter* species.
190
191 **Children with Crohn's disease (CD)**
192 Studies of faecal samples [33, 34] and mucosal biopsy specimens [35, 36] from
193 CD patients demonstrated that the gut microbiota composition of these subjects was
194 quite different from that of healthy individuals (Table 3). A reduction in the number of
195 some bacterial species in association with an increase in the number of other species is
196 almost always detected. A lower proportion of *Firmicutes* and an increase in
197 *Proteobacteria* was evidenced in most cases [37]. A disappearance of *Faecalibacterium*
198 and *Roseburia* and increase in *Enterobacteriaceae* and *Ruminococcus gnavus* was
199 demonstrated [37]. The extent of dysbiosis was associated with severity of
200 inflammation. However, most of the data are difficult to interpret because studies
201 included patients with chronic disease and previous treatments that likely modified the
202 original gut microbiota composition.
203 Studies in children with new onset disease prior to therapy initiation are more
204 indicative of the microbiota modifications that are associated with the development of
205 CD and whether a direct relationship exists between type and degree of dysbiosis and
206 disease severity. Lewis et al. examined 90 subjects < 22 years of age with active
207 disease, defined as a Paediatric Crohn's Disease Activity Index (PCDAI) score greater
208 than 10, and found that CD patients exhibited a reduced relative abundance of
209 *Prevotella, Eubacterium, Odoribacter, Akkermansia, Roseburia, Parabacteroides,*
210 *Alistipes, Coprococcus, Dorea* and *Ruminococcus* and increased abundance of

Escherichia, Klebsiella, *Enterococcus* and *Veillonella* [39]. The differences from healthy subjects were substantial, and the existing lineages predicted CD with an 86% accuracy. Shaw et al. reported that differences in *Akkermansia, Coprococcus, Fusobacterium, Veillonella, Faecalibacterium,* and *Adlercreutzia* composition between children with CD and controls were consistent with disease severity [40]. Assa et al. investigated mucosal-associated bacteria from ileal biopsy specimens obtained at colonoscopy of 10 patients with ileal or ileocolonic new onset CD and 15 controls without mucosal inflammation [41]. They identified 117 operational taxonomic units (OTUs) that were differentially abundant between controls and patients. Most of these OTUs (approximately 70%) were enriched in CD patients and annotated as unclassified *Ruminococcaceae* family members or genera of the *Ruminococcaceae* family (*Oscillospira* or *Faecalibacterium*). The increase in negative bacteria alters the availability of protective compounds, such as SCFAs, and favours abnormal immune system activity, which increases the risks of inflammation and gut wall damage.

Evidence that exclusive enteral nutrition (EEN) very effectively induces remission and reduces relapse risk, especially in paediatric patients, further highlights the relevance of gut microbiota composition in conditioning CD development and course [42, 43]. EEN is based on the administration of a liquid diet using elemental or polymeric formulae, which is exclusively administered over a prolonged period of up to 12 weeks. EEN is effective in approximately 75% of treated children, and it is superior to corticosteroids (CS). A propensity score analysis demonstrated that EEN was superior to CS for inducing remission (p=0.05) and tended to superiority for height Z score (p=0.055) [44]. Several studies demonstrated that EEN was accompanied with

significant modifications in gut microbiota with reduced proinflammatory microbial components and harmful microbial metabolites [45-51]. The most common finding was that EEN was associated with increased microbiota diversity. OTUs positively or negatively correlated with faecal calprotectin (FCP) levels, which decreased during EEN treatment [52]. Lewis et al. reported a change in microbiota composition within 1 week of EEN therapy in children with active CD, and it moved significantly farther from the composition of the microbiota centroid of the healthy controls [38]. However, children with FCP levels < 250 mg/g (i.e., responders) were closer to the centroid of the healthy controls than non-responders ($p = 0.003$). Notably, partial enteral nutrition with an ad libitum diet was not clinically effective, which suggests that the exclusion of table foods was the primary determinant in changing the gut microbiota and perhaps mediating the increased effectiveness of EEN. An increased relative abundance of Gram-positive bacteria within the phylum *Firmicutes* and decreased relative abundance of the genera from the phylum *Proteobacteria* was observed when EEN was not associated with changes in the gut microbiota diversity of paediatric CD patients [53, 54]. The alterations in gut microbiota that occur during EEN are strictly associated with the course of CD. Kaakoush et al. reported CD remission when the number of OTUs decreased during EEN, but relapses appeared when the OTUs increased after EEN completion [55]. Some data indicate that the initial microbiota composition predicts the response to EEN. Nonhuman genome in the faeces of children treated with EEN was investigated [56]. Metagenomic data were obtained using next-generation sequencing, and nonhuman reads were mapped to the Kyoto Encyclopedia of Genes and Genomes pathways, where possible. Eight pathways were identified with an expected false-

positive rate no larger than 1 in 10. Data were divided into 3 groups according to previous positive or negative connections with IBD or known as important in innate immunity and immunoregulation. CD patients and healthy subjects were different because their gut microbiota exerted different actions in xenobiotic and environmental pollutant degradation, succinate metabolism, and bacterial proteins involved in cell protection. Children with good and persistent responses to EEN possessed pathways that were significantly more similar to healthy controls than poor responders. Dunn et al. reported more detailed results of bacteria that were predictive of EEN response [57]. They investigated the composition of gut microbial community in children with CD with sustained remission after 12 weeks of EEN, children with early relapse after the same treatment and a group of healthy controls. These authors demonstrated that microbial diversity was lower in CD patients than controls, and it was lowest in patients who did not achieve sustained remission than patients who exhibited a persistent clinical response. The prevalent community in these patients was rich in *Akkermansia muciniphila* and *Bacteroides* and limited in Proteobacteria. In contrast, Proteobacteria were prominent in children with early recurrence after EEN. The differences between gut microbiota composition were so great that an 80% accuracy in differentiation between responders and non-responders was observed.

The use of biologicals in children with CD provide indirect evidence of the relevance of gut microbiota in CD. Antagonists of tumour necrosis factor α (TNFα) are recommended in moderate and severe paediatric CD who do not respond to EEN and steroids [43]. Administration of these antagonists modifies gut microbiota to resemble the microbiota after EEN. Kohlo et al. compared 32 treated children with 26 healthy

controls [58] and found that the microbial diversity and similarity to the microbiota of controls increased in children with good clinical and laboratory responses during anti-TNFα administration but not in children without response ($p<0.01$). Six groups of bacteria (*Bifidobacterium, Clostridium colinum, Eubacterium rectale*, uncultured *Clostridiales*, *Vibrio* and *Streptococcus mitis*) were related to treatment response, and the abundance of these bacteria at the genus level distinguished responders from non-responders. The abundance of two other bacterial groups, *Clostridium sphenoides* and *Haemophilus* spp., at the genus level was precisely associated with the medium-term (3 months) outcome. Wang et al. demonstrated similar results in a smaller cohort of children with CD and controls (11 and 16, respectively) [59]. All of the children with CD in this study responded to therapy, but only some of the children reached a sustained remission. The authors analysed the changes in microbiota after infliximab therapy and noted an increase in taxa with the ability to produce SCFAs, including *Anaerostipes, Blautia, Coprococcus, Faecalibacterium, Lachnospira, Odoribacter, Roseburia, Ruminococcus*, and *Sutterella*, in all patients. However, some of these taxa were increased in all children but others, including *Faecalibacterium prausnitzii,* were increased only in responders.

USE OF PREBIOTICS AND PROBIOTICS IN PAEDIATRIC CROHN'S DISEASE

If dysbiosis and CD are strictly related, then it is not surprising that prebiotics and probiotics are logical options in the treatment of this disease.

Prebiotic use

Prebiotics are food ingredients that are not digested or absorbed in the upper intestinal tract, but are fermented by gut microbiota in a selective manner and promote a relevant increase in specific bacteria, primarily bifidobacteria and lactobacilli, which confer health benefits to the host [60]. Prebiotics are generally carbohydrates, and inulin-type fructans and galacto-oligosaccharides (GOS) are the most common. Several studies reported that prebiotic administration is associated with some of the effects of probiotics administration, such as an increase in immunoregulatory interleukins, reduction in pro-inflammatory interleukins, increase in short-chain fatty acid production, and reduction in luminal pH. Local inflammation is prevented or reduced because of the colonization with acid-sensitive enteropathogens. Mucosal integrity is favoured [61].

Probiotic use

Probiotics are bacteria that exert a beneficial effect on gut structure and function because they possess anti-inflammatory activity and enhance the gut barrier. Several mechanisms were suggested to explain the potential therapeutic role of these bacteria. *In vitro* and *in vivo* studies indicate that probiotics, particularly lactic acid bacteria, exhibit a significant antioxidant potential [62]. Production of reactive oxygen species (ROS) is one cause of several gastrointestinal diseases, and probiotics exhibit a positive effect on CD course. Lactic acid bacteria reduce intestinal ROS, increase the gut concentration of antioxidant enzymes such as superoxide dismutase and glutathione and protect DNA from oxidative damage [63-65]. Probiotics reduce inflammation via improvement of intestinal barrier function and activation of the innate immune response. Supplementation with *Lactobacillus rhamnosus* GG increases the

height of pig intestinal villi and induces mucin formation, thereby protecting the intestine from pathogen invasion [63]. Moreover, probiotics can be recognized by TLRs and exhibit a strong immunomodulatory activity. *Lactobacillus plantarum* Lp91 down-regulated the expression of important pro-inflammatory cytokines in a colitis mouse model, such as TNF-α and COX2, and up-regulated the production of major anti-inflammatory cytokines, including IL-4 and IL-6 [66]. Probiotics stimulate the synthesis of defensins, which are important in the prevention of bacterial overgrowth. Reduced expression of defensins negatively affects gut microbiota composition and induces inflammation [67]. *In vitro* studies found that several *Lactobacillus* strains and VSL#3, as a probiotic cocktail of four lactobacilli, induced the secretion of the human β-defensin-2 peptide, which suggests that selected probiotics strengthen intestinal barrier functions [68]. Probiotics protect gut structure and function and global health via influencing the secretion of intestinal microflora enzymes, such as β-glucuronidase, reducing the gut content of intestinal toxic and mutagenic compounds [69], and producing short-chain fatty acids, which favours regulatory T-cell production and beneficial metabolic effects [70].

However, results on the administration of prebiotics and probiotics in CD patients seem generally unsatisfactory. Experience with prebiotics is limited, and no study evaluated children. A pilot study in 10 adult patients with moderately active CD who received 15 g/day fructo-oligosaccharides (FOS) for 3 weeks demonstrated that this supplementation was associated with a relevant reduction in disease activity and a significant increase in faecal bifidobacteria concentrations [71]. A modification of mucosal dendritic cell function was evidenced [71], but a subsequent adequately

powered study did not confirm these findings. Benjamin et al. performed a randomized double-blind placebo-controlled trial in 103 patients with active CD who were treated with 15 g/day of FOS or placebo for 4 weeks [72]. No significant difference in the number of patients who achieved a clinical response between the FOS and placebo groups was evidenced (p=0.067). No differences in faecal concentrations of bifidobacteria or *Faecalibacterium prausnitzii* between groups were detected after a 4-week intervention. Notably, more patients who received FOS (14 [26%] vs 4 [8%]; p=0.018) withdrew before the 4-week end point. This result confirms previous evidence in patients with irritable bowel syndrome treated with prebiotics. High doses of oligosaccharides may produce negative effects because of excessive luminal gas production following the fermentation of non-digestible carbohydrates [73].

Poorly satisfactory results were also obtained with probiotics. Most of the data were collected in adults using different probiotics, primarily lactobacilli, bifidobacteria, *Saccharomyces, Escherichia coli*, and “VSL#3 (a mix of lactobacilli and bifidobacteria). A meta-analysis of the randomized controlled trials published through March 2013 and including some children reported that probiotics were completely ineffective in CD independently of the probiotic used, but these agents were effective in UC [74]. Response rates were similar in patients who received probiotic supplementation and controls treated only with standard therapy using the ability of probiotics to induce remission of an active episode and maintenance of remission. Similar results were obtained when the importance of probiotics in maintaining remission was evaluated [74]. Bousvaros et al. performed a randomized, placebo-controlled trial to evaluate whether the addition of *Lactobacillus rhamnosus* strain GG (LGG) to standard therapy prolonged

372 remission in children with CD [75]. The median time to relapse was 9.8 months in the
373 LGG group and 11.0 months in the placebo group (p=0.24). The incidence of relapse in
374 the two years of follow up was 31% and 17%, respectively (p=0.18). Non-satisfactory
375 results were also reported in a very recent meta-analysis, which confirmed that
376 probiotics effectively induced or prolonged remission in UC patients but did it was not
377 substantially effective in children with CD. However, a trend for efficiency was
378 evidenced with the combination of *Saccharomyces boulardii*, *Lactobacillus* and VSL#3
379 probiotics (p=0.057) [76].

380 Faecal microbiota transplantation (FMT) in combination with prebiotics and
381 probiotics was also suggested for the treatment of CD. FMT is the transfer of faecal
382 material from a healthy donor to a patient to increase intestinal microbial diversity and
383 re-establish the composition that is generally found in healthy subjects [77]. Significant
384 changes in the structure and function of the gut microbiota were demonstrated in
385 subjects who received FMT. Paramsothy et al. showed that *Barnesiella* spp.,
386 *Parabacteroides* spp., *Clostridium* cluster IV, *Ruminococcus* spp., *Blautia* spp., *Dorea*
387 spp. and *Clostridium* cluster XVIII were significantly increased after FMT [78]. Similar
388 results were obtained by Moayyedi et al. [79] and Rossen et al. [80], who reported
389 positive results when the gut microbiota of FMT subjects was enriched with
390 *Ruminococcus* and *Clostridium* cluster IV and XIVa. Positive effects such as these are
391 supported by the findings that in experimental animals, *Clostridium* clusters XIVa, IV
392 and XVIII activate Treg cells and evoke an anti-inflammatory immune response [81],
393 and early inoculation of *Clostridia* species protects from colitis [82]. FMT produced
394 satisfactory results in refractory or frequently recurrent *Clostridium difficile* infection

(CDI) [83]. Several studies reported that FMT produced high rates of resolution of CDI-related diarrhoea (70%-90%) associated with a significant increase in microbial diversity, and independent of the mode of administration (oral capsules, enemas, and duodenal infusions). The positive impact on CDI prompted a surge of interest on the potential use of FMT as a treatment for all other diseases in which modifications of gut microbiota play a role in pathogenesis and severity, including CD. However, the results in patients with CD were not as satisfactory as the results with CDI, and contrasting results in adults were reported. Vermeire et al. reported no improvements within 8 weeks of treatment in 6 patients with refractory diseases [84]. In contrast, Vaughn et al. performed an uncontrolled open-label study in 19 patients with active CD and reported that 58% of these patients exhibited relevant clinical improvement within 12 weeks of treatment, with increases in gut microbiota diversity and quality-of-life scores [85]. A recent meta-analysis reported that the pooled proportion of patients with CD who achieved clinical remission was 52% (95% confidence interval [CI]: 31%-72%) [86]. However, data collected in children are very few and inconclusive. Analyses of reported cases suggest that the results of FMT in CD patients are strictly dependent on the route of administration and on its duration. A case series of FMT administration via nasogastric tube in 9 paediatric CD patients revealed that most patients (7/9) reached remission, which was indicated as a PCDAI <10 within 2 weeks after treatment, and 5/9 maintained remission until 12 weeks [85]. However, nasogastric tube administration was useless in patients with colonic CD, who benefited from an administration via colonoscopy. FMT administration should be protracted and requires multiple administrations in CD. This may be difficult in some paediatric cases, especially if the

418 administration requires colonoscopy and necessary sedation. A precise analysis of the
419 donor's and the recipient's microbiota is needed to avoid possible complications of the
420 introduction of potential pathogens into the patient's gastrointestinal tract.
421
422 **CONCLUSIONS**
423 Studies carried out *in vitro*, in experimental animals and in humans indicate that
424 gut microbiota are key actors in CD's pathogenesis. Development and course of CD of
425 children seem strictly associated with gut dysbiosis. However, although it well
426 established that some bacteria seem to exert a protective activity, whereas other
427 bacteria are associated with increased risk of gut wall damage, results of studies are not
428 conclusive and no definitive suggestion on the best approach to treat CD through
429 modifications of gut microbiota composition can be made. On the other hand, attempts
430 to modify gut microbiota composition in patients with CD have reported contrasting
431 results. Further studies are needed in order to identify which is the real role of different
432 bacterial taxa, genera and species in conditioning wall damage and which prebiotic
433 and/or probiotic alone or in combination can be really effective, how long they have to
434 be administered and whether or not they have to be associated with anti-inflammatory
435 and immunosuppressive drugs. The new techniques developed in metagenomics allow
436 us to reveal new details of microbiota composition in healthy subjects and CD
437 patients.These discoveries could potentially disclose new therapeutic options for CD
438 treatment and improve the existing treatments. Further studies are needed to facilitate
439 the diagnosis and tailor the therapy of a pathology that is an increasing burden on public
440 health.

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The authors report no declarations of interest.

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Table 1. Gut microbiota functions.

Function	Brief explanation
Metabolite production	The fermentation of complex carbohydrates results in the production of short-chain fatty acids (SCFAs), which are involved in many cellular processes, metabolic pathways, the enhancement of the gut barrier function and regulation of the immune system and inflammatory response [11-15]
Vitamin production	Microbiota synthesizes essential vitamins that humans are incapable of producing (e.g., vitamin B12, vitamin K); dysregulation results in metabolic pathologies, such as obesity and type 2 diabetes mellitus [11-15]
Influence on epithelial homeostasis	Microbiota promotes the epithelial integrity by influencing the turnover of the epithelial cells and modulating mucus properties [16-18]
Development of the immune system	Both intestinal mucosal defences and systemic immune system are modulated by microbiota, resulting in a greater protection against infections and against inflammatory diseases [19-23]
Influence on pathogen colonization	Microbiota competes with pathogens for the attachment sites and for the nutrients and produces antimicrobial substances [24-29]

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716 **Table 2. Factors that modify microbiota composition**
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Factor	Brief explanation
Mode of delivery	Vaginally delivered infants show elevated presence of lactobacilli, whereas the microbiota of infants delivered via C-section are richer in Clostridium species [32]
Diet	Availability of microbiota-accessible carbohydrates (MACs) that are present in dietary fibre [31] Breast-fed infants have different microbiota compared to formula-fed infants [31]
Intestinal mucus	Additional source of carbohydrates for the microbiota [33]
Sulphate compounds	Derived from diet and host mucines, influence the presence of sulphate-reducing bacteria and other species [31]
Bile acids	Their presence promotes the recovery of microbiota after an antibiotic therapy; their reduction results in the expansion of pro-inflammatory species [33]
Host immune system	Limited effect, counters the opportunistic invasion of human tissues [30]
Antimicrobials	Both administered and host-derived [31]

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723 **Table 3. Modifications of human microbiota in Crohn's disease.**
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Microbiota modification
Decrease in biodiversity [33, 34, 56, 58]
Modifications in both mucosal and luminal flora [35, 36]
Decrease in Firmicutes *phylum* [37]
Modifications of the levels of *Faecalibacterium prausnitzii* (discordant data among studies) [37]
Increase in Bacteroidetes *phylum* [37, 53-55]
Increase in Bacteroidetes *phylum*, particularly *Enterobacteriaceae* and especially *Escherichia coli* [37, 39, 40]
Decrease production of short-chain fatty acids (SCFAs) [43, 44, 59]
Increased nitrogen flux [45-51, 57]
Disrupting of molecular pathways influencing immune response and barrier integrity [38, 41, 43, 52]

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