Chapter 7
Role of Gut Microbes in Celiac Disease Risk and Pathogenesis

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Introduction

Celiac disease (CD) is a chronic enteropathy triggered by wheat gluten (e.g., gliadins) and other cereal-related proteins in genetically predisposed individuals. Typical cases of CD often appear in early childhood soon after the first exposure to dietary gluten, but the disease is also being increasingly diagnosed in late adulthood, suggesting that gluten intake is not the only triggering factor [1, 2].

The etiology and pathogenesis of the disease is strongly associated with Human Leukocyte Antigen (HLA) genes, encoding the HLA-DQ2 (HLA-DQ2.5 and HLA-DQ2.2) and HLA-DQ8 heterodimers, involved in antigen presentation and T-cell activation. These genetic factors are necessary for the disease to develop but not sufficient, since they are also present in ~35% of the general population and only a small percentage (3–5 %) develops the disease [3, 4].

To date, gluten is the only known environmental factor to play a direct causal role in CD. As discussed by Ludvigsson et al. in Chap. 3 and elsewhere in this book, epidemiological studies report that mode of delivery at birth, milk-feeding type and incidence of infections and antibiotic intake, which may also influence the gut ecosystem, are some other factors influencing the risk of developing CD [5–8]. In early stages of life, interactions between gut microbiota and innate and adaptive immunity play a crucial role in influencing T effector- and regulatory-cell balance and the development of tolerance towards dietary antigens [9, 10]. In this context, the

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incidence of gastrointestinal viral infections, which generate Th1-protective responses, has been investigated as a possible factor contributing to CD risk [11]. Prospective studies in infants at risk of CD are also underway to find out possible relationships between their gut colonization pattern and CD onset [6, 12]. In addition, observational studies in children and adults have revealed alterations in the gut microbiota composition of subjects with active CD (symptomatic and untreated) and non-active CD (non-symptomatic after following a gluten-free diet) compared to that of control subjects, which could contribute to the disease pathogenesis [5].

Currently, CD is among the most prevalent chronic digestive disorders, but the only effective therapy for CD patients is life-long adherence to a strict gluten-free diet. Complying with this dietary recommendation is difficult because gluten is present in most processed food, and patients are continuously exposed to gluten. Also, a small proportion of patients (~5 %) do not experience improved clinical symptoms on a gluten-free diet and can be diagnosed with refractory CD [13–15]. Therefore, there is a need to identify modifiable factors that contribute to CD risk and pathogenesis in order to make progress in the identification of complementary strategies to improve the quality of life of patients and to prevent the disease from developing in populations at risk.

Influence of Milk-Feeding Type in Gut Microbiota and Risk of CD Development

Dietary influences are particularly relevant at early stages of development, when the infant's gut and immune system are immature, because of their possible contribution to either the development of oral tolerance to innocuous antigens and commensal bacteria or to increasing overreactions and disease risk. Colonization of the intestine starts immediately after birth and represents the main environmental stimulus for immune system maturation. This process depends, among other factors, on the type of delivery, type of milk-feeding, and, possibly, host genotype (Fig. 7.1) [16].

Breast-feeding is an environmental factor that seems to protect or at least delay CD development; this effect can be due to its immune and other biologically active components [17], as well as to its effect on the intestinal microbiota composition [18]. Breast-fed children exhibit higher prevalence of bifidobacteria; meanwhile, formula-feeding favors the colonization of a more heterogeneous microbiota that is more similar to that identified in the adult population [19, 20].

Studies using real-time PCR and denaturing gradient gel electrophoresis (DGGE) in a cohort of 164 infants at risk of developing CD have also demonstrated that both milk-feeding type and HLA-DQ genotype influence the intestinal microbiota [6, 12]. The microbiota of infants at high risk of developing CD showed reduced numbers of *Bifidobacterium* spp. and, particularly, of *B. longum*. Although breast-feeding reduced the genotype-related bifidobacterial alterations, these were not

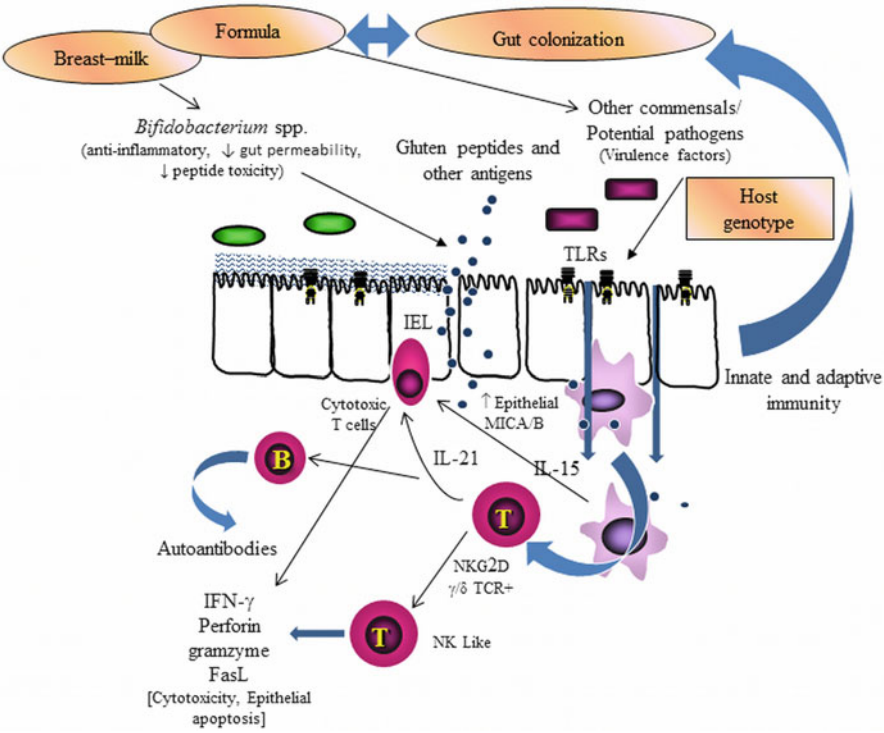

Fig. 7.1 Schematic representation of different factors influencing gut microbiota composition and its possible role in CD pathogenesis and risk of developing CD

completely normalized, suggesting that both factors (milk-feeding type and HLA-DQ genotype) influence the composition and numbers of *Bifidobacterium* spp. [6]. Formula-fed infants at high genetic risk of developing CD also showed increased numbers of the *Bacteroides fragilis* group but not breast-fed infants, suggesting that the effect of breast-feeding on colonization of this bacterial group is stronger than the possible effect of the CD genotype [6].

In infants at high risk of developing CD, numbers of *Staphylococcus* spp. were increased in both breast- and formula-fed groups, suggesting a major role of the HLA-DQ genotype in defining the colonization of this bacterial group [6]. In another study with a subgroup of infants [12] from this cohort and using DGGE, increased *B. vulgatus* prevalence was associated with the high-risk genotype, while increased *B. uniformis* prevalence was associated with both the low-risk genotype and breast-feeding. Overall, breast-feeding seems to attenuate the differences in microbiota related to the HLA-DQ genotype, which could partly explain its protective effect against CD development reported in previous epidemiological studies.

Role of Infections in Risk of CD Development

Infectious agents have been suggested to contribute to determining the risk of developing CD by antigen mimicry molecular mechanisms, increasing intestinal permeability or boosting a protective immune response similar to that caused by dietary gluten peptides. However, while some epidemiological studies suggest that infections increase the risk of CD development [9, 21], others support the opposite hypothesis [22]. The study by Plot et al. [21] investigated the association of CD with five major infectious agents, including *Toxoplasma gondii*, rubella virus, cytomegalovirus, *Treponema pallidum,* and Epstein–Barr virus based on the detection of serum-specific antibodies. This study reported lower prevalence of IgG antibodies to these infectious agents in CD patients compared to healthy subjects, indicating that these infections can protect against CD development. In contrast, a study in 3,392 Swedish infants, who ultimately developed CD, compared to healthy infants, reported that neonatal infections were the main risk factor for developing CD [9].

Other studies have also supported the hypothesis that an autoimmune response could be caused by cross-reactivity between a gluten peptide epitope within the alpha-gliadin and immunologically similar epitopes in the infectious organism, in particular the Elb protein of the adenovirus 12 [22]. However, this relationship was not consistently confirmed by measuring specific IgG antibodies for the E1d protein in the sera of children with CD [23, 24]. An association between hepatitis C viral infection and CD onset has also been proposed. This hypothesis was based on the fact that prevalence of CD in patients with chronic liver disease was found to be 15 times higher than in the general population [25], and that 5 % of autoimmune diseases diagnosed in these subjects was CD [26]. Nevertheless, other studies have not reported an increased prevalence of CD in patients suffering hepatitis C [27]. Increased incidence of rotavirus infection has also been linked to increased risk of CD autoimmunity in a prospective study including 1,931 children carrying CD HLA risk alleles [11]. In some particular cases, gastrointestinal infections caused by *Campylobacter jejuni* [11] or *Giardia lamblia* [28] have also been associated to CD onset based on their simultaneous diagnosis.

Intestinal Dysbiosis and CD Pathogenesis

CD has been associated with alterations in the intestinal microbiota composition (intestinal dysbiosis) in several observational human studies in children and adults (Table 7.1). The microbiota of CD children showed an increased ratio of Gram-negative to Gram-positive bacteria. In particular, CD patients showed reduced numbers of *Bifidobacterium* spp. and *B. longum* and increased numbers of *Bacteroides* spp. in stools and duodenal biopsies, analyzed by molecular quantitative methods (e.g., FISH and real-time PCR), compared to controls [29, 30]. Enterobacteria and staphylococci numbers were also higher in untreated CD patients than in controls,

Table 7.1 Summary of studies reporting associations between gut microbiota composition and celiac disease

Study group	Sample	Technique	Results	References
Children with active CD and non-active CD[a]	Duodenal biopsy	FISH coupled with flow cytometry	↑ Total and Gram-negative bacteria in patients with active CD ↑ *Bacteroides-Prevotella* and *E. coli* in patients with active CD ↓ *Lactobacillus+Bifidobacterim/Bacteroides-Prevotella +E. coli* in patients with active and non-active CD	[29]
Children with active CD and non-active CD	Feces and duodenal biopsies	Real-time PCR	↑ *Bacteroides* and *Clostridium leptum* groups in feces and biopsies of patients with active and non-active CD ↑ *E. coli* and *Staphylococcus* spp. in feces and biopsies of patients with active CD ↑ *Bifidobacterium* spp. and *B. longum* in feces of both groups of CD patients and in biopsies of patients with active CD	[30]
Children with active CD and non-active CD	Duodenal biopsy	TTGE	↑ Diversity (number of bands) in patients (with active plus non-active CD) ↑ *Bacteroides vulgatus* and *E. coli* in patients (with active plus non-active CD)	[31]
Children with active CD (newly diagnosed and challenged with gluten) and non-active CD	Jejunal biopsies	SEM	↑ Rod-shaped bacteria in patients with active and non-active CD than in controls	[32]
Children with active CD (newly diagnosed and challenged with gluten) and non-active CD	Distal duodenum/ proximal jejunum biopsies	SEM 16S rRNA gene sequencing	No differences by SME in samples collected from 2004 to 2007 ↑ *Haemophilus* spp. and ↓ *Neisseria polysaccharea* in patients (with active plus non-active CD) than in controls in samples collected from 2004 to 2007 ↑ Unclassified *Clostridiales, Actinomyces graevenitzii, Prevotella spp., Bacteroidetes, Firmicutes* and *Fusobacteria* in SME+biopsies than in SME-biopsies collected in the Swedish CD epidemic (1985–1996)	[33]

(continued)

Table 7.1 (continued)

Study group	Sample	Technique	Results	References
Adults patients with active and non-active CD	Fecal samples	DGGE	↓ Diversity *Lactobacillus* and *Bifidobacterium* species in patients with non-active CD ↑ *Bifidobacterium bifidum* patients with active CD than in healthy adults	[34]
Children with non-active CD	Duodenal biopsy and fecal samples	DGGE Plate counts	↑ Diversity of Eubacteria in duodenal samples of CD patients and differences in DGGE profiles compared with controls ↓ *Lactobacillus, Enterococcus*, and *Bifidobacterium* plate counts in fecal samples of CD patients than in controls ↑ *Bacteroides, Staphylococcus, Salmonella, Shigella*, and *Klebsiella* plate counts in CD than in controls	[35]
Healthy adults before and after GFD	Fecal samples	FISH Real-time PCR	↓ *Bifidobacterium, Clostridium lituseburense*, and *Faecalibacterium prausnitzii* proportions after GFD by FISH ↓ *Bifidobacterium, Lactobacillus*, and *Bifidobacterium longum* and ↑ *Enterobacteriaceae* and *E. coli* after GFD by real-time PCR	[36]
Children with active CD, adults with non-active CD	Small intestinal biopsy	Real-time PCR	No differences in numbers or prevalence of groups analyzed (*Bifidobacterium, Bacteroides-Prevotella, Bacteroides, Streptococcus*, and *Lactobacillus*)	[37]
Children with active CD	Small intestinal biopsy	IS-pro, 16S-23S interspacer region-based profiling	No differences in diversity and composition	[38]
Adults with active CD with different symptomatology	Duodenal biopsy	DGGE	↓ Diversity in CD patients with gastrointestinal symptoms and anemia than those with dermatitis herpetiformis (DH) ↑ Proteobacteria in CD patients with gastrointestinal symptoms in comparison with those with DH and controls	[39]

[a]Patients with active CD were newly diagnosed cases on a normal gluten-containing diet (or in a few cases challenged with gluten) and patients with non-active CD were symptomatic-free treated with a gluten-free diet

but the differences were restored in CD subjects on a long-term gluten-free diet [30]. Another research group also analyzed the mucosa-associated microbiota of children with CD, before and after following a gluten-free diet, by temporal temperature gradient gel electrophoresis (TTGE) reporting that prevalence of *Bacteroides vulgatus* and *Escherichia coli* in CD patients was higher than in controls [31], partially in agreement with previous studies [30]. In adults it was reported that rod-shaped bacteria were frequently associated with the mucosa of CD patients (both active and treated with a gluten-free diet), but not with controls, as determined by scanning electron microscopy [32].

A further retrospective study of the biopsy samples of these CD patients by 16S rDNA sequencing identified *Clostridium* spp., *Prevotella* spp., and *Actinomyces* spp. as the main components of the small intestinal microbiota of children born during the Swedish CD epidemic in 2004–2007, concluding that these microbial groups could have been risk factors contributing to the increased disease incidence [33]. Nevertheless, these bacteria have not been found in new CD cases, suggesting that initial associations were casual, while causality between specific intestinal bacteria and CD onset has yet to be proven. Additional studies of the microbiota of adult CD patients by DGGE clustered the dominant microbial communities of healthy individuals together and separated from those of untreated CD patients [34]. Adult CD patients treated with a gluten-free diet also showed different DNA profiles and/or reduced diversity of *Lactobacillus* spp. and *Bifidobacterium* spp. [34, 35]. In agreement, quantitative analyses of the microbiota of healthy subjects under a gluten-free diet indicate that the diet per se reduced the numbers of *Lactobacillus* spp. and *Bifidobacterium* spp. and is partly responsible for the differences found between treated CD patients and controls [36]. The analysis of metabolites derived from intestinal microbiota activity has also revealed significant differences between treated CD patients and healthy adults, suggesting metabolic signatures of CD [34, 35]. In the most recent study, the duodenal microbiota of CD patients, stratified according to the disease manifestation for the first time, has been analyzed by DGGE [39]. The findings indicate that patients with gastrointestinal symptoms have differences in the microbiota structure (dominated by Proteobacteria) in comparison with those that have dermatitis herpetiformis and controls, suggesting that intestinal microbiota play a role in the manifestation of CD [39].

The isolation and identification of clones belonging to some bacterial groups associated with CD and characterization of their virulence-related genes have recently led to the identification of more specific differences between CD patients, with active and non-active disease, and controls [40, 41; Table 7.2]. *E. coli* clones belonging to virulent phylogenetic groups (B2 and D) isolated from untreated and treated CD patients carried a higher number of virulence genes encoding P fimbriae, capsule K5, and hemolysin than those isolated from healthy controls [40]. The isolation and identification of clones belonging to the genus *Staphylococcus* also revealed that *S. epidermidis* carrying methicillin-resistant genes (*mecA*) was more abundant in both treated and untreated CD patients than in controls [42]. Of the *Bacteroides* spp. isolated and identified from stools, *B. fragilis* carrying genes

Table 7.2 Summary of pathogenic features identified in gut bacteria isolated from celiac disease patients

Pathogenic bacteria	Virulence factor (gene) analyzed	Results	References
Escherichia coli	Phylogenetic classification in commensal (A + B1) and virulent (B2 + D) groups	The four phylogenetic groups were equally distributed in healthy control children.	[40]
	Type 1 fimbriae (*fimA*), P fimbriae (*papC*), S fimbriae (*sfaD/E*), Dr haemagglutinin (*draA*), haemolysin (*hlyA*), capsule K I (*neuB*), capsule k5 (*KfiC*), aerobactin (*iutA*)	↑ Virulent group B2 in patients with active CD ↑ Virulent group D in patients with non-active CD ↑ Carriage of genes coding for P fimbriae, capsule k5 and haemolysin in patients with active and non-active CD	
Bacteroides spp.	Metalloproteases (*bft* and *mpII*)	↑ *B. fragilis* isolates carrying genes coding for metalloproteases in patients with active and non-active CD	[41]
Staphylococcus spp.	Adhesion (*atlE* and *fbe*), cell aggregation (*icaD*), global regulatory (*agr* and *sar*), and methicillin-resistant (*mecA*)	↑ *Staphylococcus epidermidis* coding for the *mecA* gene and simultaneously for both the *mecA* and *atlE* genes in patients with active and non-active CD	[42]

encoding metalloproteases was more abundant in CD patients (treated with a gluten-free diet and untreated) than in healthy controls [41]. Taken together, these studies demonstrate that imbalances in gut microbiota of CD patients do not occur as a consequence of inflammatory processes associated with active phases of CD alone, and could primarily contribute to disease onset and pathological manifestations, although effects of the gluten-free diet cannot be ruled out [41].

In contrast, two other studies reported that the duodenal mucosa-associated microbiota was similar in untreated CD patients and controls by using a DNA profiling method based on amplification of the 16S-23S interspacer gene region [38] and quantitative reverse transcription PCR [37], which contradicts evidence supported by the majority of studies. The question of whether small intestine bacterial overgrowth (SIBO) is more prevalent in CD or when present is a contributor to refractory sprue was addressed in only one study reported by Chang and Green who found no benefit for the use of Rifaximin in CD patients with SIBO having poorly responsive disease [43].

Several mechanisms by which intestinal dysbiosis may influence pathogenesis of CD have been proposed, including the generation of toxic and immunogenic

peptides from gliadins by their proteolytic activities [41], alteration of the gut barrier function and the composition of the glycocalix modifying bacterial adhesion and possible peptide translocation [44, 45], and activation of inflammatory cytokine production [46].

Defects in intestinal barrier function favor the access of gliadins to the lamina propria and its interaction with infiltrated lymphocytes and antigen presenting cells. Gliadins impair intestinal integrity through alterations of proteins (zonulin, occludin, cadherin, and claudins) involved in tight junctions between intestinal cells [47, 48]. This response occurs in parallel with the production of pro-inflammatory cytokines such as TNF-α and IL-1 β, exerting a negative feedback and increasing intestinal permeability [49, 50].

Patients with CD present increased production of type 2 mucin (MUC2), in comparison to patients on a gluten-free diet [32], associated with metaplasia of goblet cells and intestinal mucosal atrophy. Recent data from animal trials using intestinal loops also demonstrated the ability of potentially harmful enterobacteria (*E. coli* CBL2) and pathogens (*Shigella*) to reduce the number of goblet cells producing mucus and to increase intestinal permeability, leading to gliadin translocation to the lamina propria [46]. These enterobacteria also exert negative effects by reducing the production of a tissue inhibitor of a metalloprotease (TIMP-1) and increasing the secretion of vascular endothelial growth factor in intestinal loops [46]. In vitro studies in peripheral blood mononuclear cells (PBMCs) have indicated that these enterobacteria induce the production of IL-12 and/or interferon-γ, associated with increased expression of molecules HLA-DR and CD40, which could boost the adverse response to gluten [51]. In contrast, none of the effects described above have been observed for *Bifidobacterium bifidum* CECT 7365 and *Bifidobacterium longum* ATCC 15707 [46, 51]. *B. bifidum* CECT 7365 and *B. longum* CECT 7347 have also shown to exert positive effects increasing IL-10 and decreasing interferon-γ production by PBMCs [52].

Activation of toll-like receptors (TLRs), whose response may be enhanced by MHC-II molecules [53], play an important role in the recognition of microbial and other antigens, activation of different signaling pathways, expression of genes, and production of cytokines [54] regulating innate immunity (Fig. 7.1). Analyses of biopsies from CD patients have reported an increased expression of TLR2-sensing bacterial lipopeptides [37, 55–57] and TLR9-sensing bacterial DNA [37]; meanwhile, opposite results concerning TLR4 expression-sensing lipopolisaccharide from Gram-negative bacteria have been published [37, 55–57]. It can be hypothesized that increased expression of TLRs in the CD mucosa could amplify signaling through interactions with intestinal bacterial antigens and act together with gluten to immune activation. Activation of TLR3, for example, by virus, is known to induce cytokine production through a signaling pathway dependent on MyD88, which activates production of molecules related to type 1-like interferons that could contribute to insaturation of autoimmune diseases such as CD [58]. Moreover, wheat components such as the alpha-amylase/trypsin inhibitors like CM3 and 0.19, present in the globulin fraction of cereal grains, have also been identified as strong activators of TLR4 in monocytes, macrophages, and dendritic cells [59].

Potential Role of Probiotics in CD

The associations between CD and intestinal dysbiosis and the role attributed to some probiotics in the regulation of the gut barrier function and immunity in several inflammatory disease models have motivated investigations into the potential protective role of specific intestinal bacteria in CD [60, 61]. It has been suggested that these bacteria could contribute to reducing the risk and severity of the disease by their immunomodulatory features, capacity to eliminate immunogenic peptides from gluten, and improvement of intestinal permeability and restoration of the intestinal ecosystem (see Fig. 7.1).

In vitro studies have demonstrated the ability of different bifidobacteria (especially *B. longum* CECT 7347) to hydrolyze gliadin peptides, thereby generating products with lower inflammatory potential and cytotoxicity than those generated during simulated gastrointestinal digestion in absence of bifidobacteria [62]. Some other components of the *Rothia* genus from the oral cavity have also proved their proteolytic capacity on gluten peptides, cleaving down immunogenic epitopes, and reducing their inflammatory potential [63]. In addition, a strain of *Bifidobacterium lactis* positively counteracted harmful effects of toxic gliadin on intestinal epithelial cell culture integrity, reducing membrane ruffle formation [64].

Diverse studies indicate that specific probiotic strains can play a role in the production of chemokines and cytokines, determining Th1/Th2-cell balance and regulating oral tolerance to innocuous antigens [65]. Specific probiotic strains can also influence innate immune responses via their interaction with TLRs and antigen presenting cells, although most evidence comes from animal studies. Data from transgenic mice expressing HLA-DQ8 molecules demonstrate that maturation of dendritic cells, isolated from bone marrow of these animals, is favored by incubation with the different lactobacilli (*L. paracasei* IMPC2.1, *L. fermentum* BIO-DRL36, and *L. casei* ATCC 9595) showing strain-specific effects on TNFα production [66]. Moreover, the simultaneous administration of gliadins and *L. casei* ATCC 9595 to animals sensitized with indomethacin also boosted immune response of T (CD4+)-cells against gliadins in these transgenic mice [67]. The authors of the aforementioned study suggested the potential use of this strain as coadjutant in vaccines, favoring adaptive immunity against gluten antigens. Another study evaluated the influence of *B. longum* CECT 7347 orally administered to weanling Wistar rats, sensitized with interferon-γ, and fed gliadin to partially reproduce CD [61]. In this model, animals fed with this *Bifidobacterium* stain had lower numbers of peripheral T CD4+ and T regulatory (Treg) CD4+/Foxp3+ (forkhead transcription factor 3) cells and increased IL-10 production in jejunal sections compared to animals fed with placebo. However, human studies are required to confirm that CD patients and populations at risk of CD benefit from bifidobacteria and lactobacilli intake, evaluated preclinically to date.

In summary, scientific evidence from most human observational studies demonstrates that CD is associated with shifts in the assembly of intestinal microbiota to a state of dysbiosis that involves overgrowth of potentially pathogenic bacteria.

Although these perturbations are partially restored after adherence to a gluten-free diet and could be considered as a secondary consequence of the disease, they may alter the host-microbe crosstalk and contribute to CD pathogenesis. Furthermore, healthy infants at risk of developing CD also show alterations in the intestinal colonization pattern early in life, which suggest a primary role for the intestinal microbiota in this disorder. Altogether, findings indicate that gut microbiota and the impact of host and environmental perturbations on it could be part of the puzzling features of CD and its investigation could help to understand the disease onset and identify preventive strategies targeting the gut ecosystem.

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