Review

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Gut microbiome investigation in celiac disease: from methods to its pathogenetic role

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Abstract: Our body is inhabited by a variety of microbes (microbiota), mainly bacteria, that outnumber our own cells. Until recently, most of what we knew about the human microbiota was based on culture methods, whereas a large part of the microbiota is uncultivable, and consequently previous information was limited. The advent of culture-independent methods and, particularly, of next-generation sequencing (NGS) methodology, marked a turning point in studies of the microbiota in terms of its composition and of the genes encoded by these microbes (microbiome). The microbiome is influenced predominantly by environmental factors that cause a large inter-individual variability (~20%) being its heritability only 1.9%. The gut microbiome plays a relevant role in human physiology, and its alteration (“dysbiosis”) has been linked to a variety of inflammatory gut diseases, including celiac disease (CD). CD is a chronic, immune-mediated disorder that is triggered by both genetic (mainly HLA-DQ2/DQ8 haplotypes) and environmental factors (gluten), but, in recent years, a large body of experimental evidence suggested that the gut microbiome is an additional contributing factor to the pathogenesis of CD. In this review, we summarize the literature that has investigated the gut microbiome associated with CD, the methods and biological samples usually employed in CD microbiome investigations and the putative pathogenetic role of specific microbial alterations in CD. In conclusion, both gluten-microbe and host-microbe interactions drive the gluten-mediated immune response. However, it

remains to be established whether the CD-associated dysbiosis is the consequence of the disease, a simple concomitant association or a concurring causative factor.

Keywords: celiac disease; dysbiosis; microbiome; microbiota; *Neisseria flavescens*; next-generation sequencing.

Introduction

The human body is inhabited by a variety of microbes, bacteria and, to a lesser extent, by archaea, fungi and viruses [1]. The number of bacteria and other microbes that we host outnumber our own cells. Indeed, the first estimated microbial to human cells was 10:1 [2], which is probably an overestimate depending on the proportion of bacteria in our guts, which stretches from the mouth to the anus, and from the more or less good estimate of the number of human cells. In fact, a recent study suggested a ratio of 1.3:1 microbial to human cells [3]. Notably, there is an average of 30 trillion (10^{12}) human cells and 39 trillion bacteria in a man aged between 20 and 30 years, weighing 70 kg and 1.70 m tall [3]. The human microbiota is the ecological community of symbiotic, commensal and pathogenic microorganisms (the microbial taxa) that inhabit the surfaces and specific niches of our organism (gut, skin, mouth, etc.), whereas, the term human “microbiome” indicates the catalog of genes encoded by these microbes [4] (Table 1). In the past, most of what we knew about the human microbiota was based on culture methods, but it was estimated that as much as 20%–60% of human microbes are uncultivable [5–7], therefore previous information was limited. It was only after the development of culture-independent methods and, particularly, in the early 2000s, of next-generation sequencing (NGS) methodology [8], together with the development of powerful computational tools and subsequently of “omics” technologies (Table 1), that we were able to efficiently catalog microbes and their genes, and to establish the impact of the host-microbe interaction on human metabolism [9–11]. Microbiome changes have been linked to many complex immune-mediate diseases, among which celiac disease (CD) [12–27].

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Table 1: Main glossary in microbiome studies.

Human microbiota: The trillions symbiotic microbial cells (bacteria, viruses or eukaryotes) inhabiting mucosal surfaces of our body
Human microbiome: The collective genes encoded by the human microbiota. The microbiome is different in the different niches of our body (i.e. duodenal microbiome is different from fecal or skin microbiomes)
Prevalent genes: Non-redundant genes present in the human gut microbiome
Common genes: Genes found in the gut microbiome of at least 50% of individuals
Gut microbiome: The catalog of 3.3 million non-redundant microbial genes present in the human gut
Gut common core: About 38% of the individual's gut microbiome shared among different individuals
16S rRNA sequencing: Evaluation of the bacterial composition in a human niche (i.e. gut microbiome) based on the 16S rRNA marker (usually up to genus taxon level)
Metagenomics: Evaluation of the microbial composition in a human niche (i.e. gut microbiome) based on the whole genome sequencing of all microbes present (i.e. up to bacterial species and subspecies presence)
Metatranscriptomics: Evaluation of the microbial function in a human niche based on the whole RNA sequencing of all microbes present (i.e. gut metatranscriptomics)
"Omics" technologies: A set of methods used to investigate the interaction microbiome-host (i.e. metagenomics, metatranscriptomics, metaproteomics, metabolomics)
Dysbiosis: Imbalance of microbes inhabiting a human body niche

CD is a chronic, immune-mediated disorder that occurs at any age, and affects more women than men with a total prevalence of around 1% in Caucasians in Europe [28, 29]. CD involves both genetic and environmental factors [29]. The major contributing genes are those encoding the HLA-DQ2/DQ8 molecules [30] and, to a lesser extent, non-HLA genes [31]. Globally, genes account for 48% of the disease risk [31–33]. What is known so far about the pathogenesis of CD is that, in HLA-genetically predisposed individuals, wheat gluten proteins and proteins found in barley and rye, after ingestion, are modified by intestinal transglutaminase 2 (TG2) into highly immunogenic deamidated gluten peptides (DGP). The latter, after binding to HLA-DQ2/DQ8 molecules on dendritic cells (DCs), are presented to CD4+ T-cells thereby activating immune reactions (production of CD-specific antibodies, release of pro-inflammatory cytokines, and activation of intraepithelial lymphocytes) [34]. This succession of events induces inflammation and progressive histological changes in the small intestinal mucosa, namely an increase in intraepithelial lymphocytes (>25/100 enterocytes), crypt hyperplasia and villous atrophy [35]. Although gluten remains the major triggering factor of CD, several questions remain

unanswered. Why does CD develop at any age even years after the introduction of gluten in the diet [36]? Why has the prevalence of CD increased over time in the Western world, although gluten-containing food consumption has not increased [37]? Why do all HLA DQ2/DQ8 genetically predisposed individuals not develop the disease [38]? Conflicting results have recently been reported regarding the influence on the CD risk of the timing and the amount of gluten consumption during infancy, likely due to different study designs [39, 40]. Furthermore, although a gluten-free diet (GFD) improves mucosal lesions in CD patients, it does not correct the intestinal immune response [41]. The aforementioned observations suggest that other pathogenic factors are involved in the onset of CD. We and others reported gut dysbiosis in adults and/or children affected by CD but not in controls, which indicates that the gut microbiome is an additional contributing factor to CD pathogenesis [12–17, 19–27].

The gut microbiome, general features and its role in human physiology

Within the framework of the Human Microbiome Project, tissue samples from multiple body sites (gastrointestinal tract, mouth, vagina, skin, etc.) of 250 “normal” volunteers were analyzed to determine whether the monitoring or manipulation of the human microbiome could improve human health [42]. Subsequently, the study by Qin et al. [9], in which they examined fecal samples of 124 European individuals, was crucial in establishing the minimal gut metagenome (Table 1) and the minimal gut “bacterial” genome in terms of functions encoded by this gene set. In particular, the latter study [9], which was part of the Metagenomics of the Human Intestinal Tract (MetaHIT) Consortium Project established a catalog of 3.3 million non-redundant genes (prevalent genes) in the gut microbiome, most of which were present in only a few individuals (<20%), whereas 294,110 genes were considered “common genes” being found in at least 50% of individuals [9] (Table 1). In detail, each individual carries in their gut ~536,112 prevalent microbial genes among which ~204,056 are common genes, which indicates that about 38% of an individual's gut microbiome is shared (gut common core) (Table 1) [9].

A comparison between the human genome (~22,000 genes) [43] and diversity (~99.9% identity between individuals) [44] with the microbial genes catalog (human

gut microbiome ~3.3 million non-redundant genes) [9] and diversity (80%–90% diversity between individuals) [45] suggests that to study the microbiome together to human genome is mandatory to carry out “personalized medicine”.

As reported in Table 2, the gut microbiome plays relevant roles in human physiology: it produces vitamins, contributes to food digestion, preserves the structural integrity of the gut mucosal barrier thereby impeding the colonization of pathogens, participates in the gut-brain axis communication and primes the immune system thereby helping to maintain a healthy status [46]. However, it is not easy to define the “gut health microbiome”. In fact, the microbiome composition is influenced by several factors: host genetics, diet, drugs, lifestyle and other environmental factors. Rothschild et al. [47] recently examined approximately 1000 healthy individuals of different ancestral origin and found no significant differences between ancestries in terms of microbiome composition, which suggested that the microbiome is not closely associated with host genetics. The same authors estimated that the microbiome heritability weight was only 1.9% in a dataset of 2252 twins [48]. By contrast, significant microbiome similarities were found in first-degree relative pairs

and in pairs of genetically unrelated individuals who reported sharing a household [47]. In detail, about 20% of inter-person microbiome variability was associated with multiple environmental factors (diet, drugs, physical activity, etc.) and the body mass index [47]. Globally, microbiome composition is predominantly shaped by non-genetic factors that cause a large intra- and inter-individual variability, which makes it difficult to define what is a “healthy gut microbiota”. However, although each individual is characterized by a specific combination of bacterial taxa, gut microbiota functions are highly preserved. Therefore, from a clinical point of view, any microbial pattern associated with a healthy host status could be considered “healthy” [49].

In support of the relevance of microbiome functions, a recent study identified metagenomically abundant organisms that were inactive or dormant in the gut with being little or not expressed [50], which indicates that disease-specific microbial alterations can sometimes be detectable only at the transcriptional level. Globally, it is now evident that, besides the microbial composition (metagenomics) of the gut, also its activity (metatranscriptomics) should be evaluated (Table 1) in order to explore the role of the microbiome in human health and disease [51].

Table 2: Role of gut microbiota in human physiology.

Gut microbiota function	Metabolites produced by gut microbiota and their impact on the host
Metabolism – Host energy harvesting (i.e. unabsorbed starch and soluble dietary fiber fermentation) – Production of vitamins and acetylcholine – Bile acids metabolism	– Fermented end products: SCFAs ^a ; microbes (<i>Roseburia hominis</i> and <i>Fecalibacterium prausnitzii</i> [Firmicutes], etc.); energy substrate for the host; ATP production in colonocytes – Vitamins K, B (B5, B12) and choline metabolites; microbes (<i>Bacteroides fragilis</i> , <i>Eubacterium lentum</i> , <i>Serratia marcescens</i> , etc.); lipid metabolism; energy production; enzymatic cofactor; nervous system functioning – Production of secondary bile acids; microbes (<i>Clostridium perfringens</i> and <i>C. scindens</i>); glucose and energy metabolism; antimicrobial effects
Gut barrier integrity and permeability – Colonization resistance	– SCFAs ^a ; microbes (<i>R. hominis</i> and <i>F. prausnitzii</i> [Firmicutes], etc.); downregulation of pathogens virulence factors and modulation of pH; nutrient competition with pathogens
Host immune maturation and function – Aberrant immune-inflammatory response prevention	– Polyamines (putrescine, spermidine, spermine); indole derivatives; Microbes (<i>B. fragilis</i> [Bacteroidetes], <i>Bifidobacterium infantis</i> [Actinobacteria] and Firmicutes, etc.); enhancement of the intestinal barrier integrity (by stimulation of intercellular junction proteins synthesis), inhibition of macrophage activation and tight regulation of Treg/T _H 17 ratio
Modulation and function of the enteric nervous system (ENS) – Intestinal homeostasis	– SCFAs ^a ; Microbes (<i>R. hominis</i> and <i>F. prausnitzii</i> [Firmicutes], etc.); recognition and reaction of ENS ^a (via TLR4 ^a) to the G(–) bacterial LPS ^a ; gut-brain axis: bidirectional communication of ENS with CNS ^a regulates a variety of gastrointestinal functions via vagal pathways (exocrine/endocrine secretions, gut motility, immune-inflammatory reactions, etc.)

^aSCFAs, short chain fatty acids: butyrate, propionate, acetate and pentanoate; ENS, enteric nervous system; CNS, central nervous system; TLR4, toll like receptor 4; LPS, lipopolysaccharide.

Gut microbiome profiles in controls and in patients affected by CD

As mentioned, the gut microbiota is composed of several types of microorganisms, namely bacteria, archaea, fungi and viruses, among which bacteria are the most abundant [1]. To date, most of the microbiota studies conducted in patients affected by CD have been bacteria-centric [12–27], whereas the mycobiome and virome have also been studied as implicated in the pathogenesis of CD [52–54]. Currently, the 16S rRNA gene marker evaluation and NGS (see Supplementary material) are the most widely used techniques with which to explore the abundance and diversity of bacterial taxa composition (from phylum to class, order, family, genus) present in a human body niche or sample.

A few phyla dominate the healthy human gut: Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria and Verrucomicrobia. The first two phyla constitute about 90% of the gut microbiota [55]. *Clostridium*, *Lactobacillus* and *Enterococcus* are the most abundant Firmicutes, *Bacteroides* and *Prevotella* are the most abundant Bacteroidetes, whereas *Bifidobacterium* is the most abundant Actinobacteria [55]. Microbiota composition differs in the various parts of the gastrointestinal

tract, which is affected by pH, host secretion, substrate availability, etc. thereby resulting in a craniocaudal progressive decrease of aerobic and an increase in strictly anaerobic bacterial species [56]. Consequently, the gut microbiome profile associated with a healthy condition and/or with an inflammatory gut disease, including CD, depends on the specific niche or sample investigated, as well as on the all other factors influencing microbiota variability (Table 3).

Oral microbiome

Oral microbiota is the second most complex and abundant microbial community in the human body after the colon [42]. The microbiome associated with CD patients has also been investigated in saliva and oropharyngeal swabs, which are easy to obtain and to study [26, 27]. The rationale of studying the oral microbiome is that gluten is first processed in the mouth, consequently the oral microbiome might have an impact on the immunogenic peptides produced after this first part of the digestion process. Recently, Tian et al. [27] reported an increased abundance of Actinobacteria and a reduced presence of Bacteroidetes and Fusobacteria phyla in the oral microbiota of refractory

Table 3: Gut dysbiotic features observed in active CD patients.

Biological sample	CD associated dysbiosis	References
Feces	↑ Gram (–)/Gram (+) bacteria ratio	Sanz et al. [12]
	↓ Firmicutes (<i>Lactobacillus</i> spp., <i>Faecalibacterium prausnitzii</i> , <i>Clostridium</i> spp.)	Di Cagno et al. [13]
	↓ Actinobacteria (<i>Bifidobacterium</i> spp.)	Collado et al. [14]
	↑ Bacteroidetes (<i>Bacteroides</i> spp.)	De Palma et al. [15]
	↑ Proteobacteria (<i>E. coli</i>)	Quagliarello et al. [16]
	↑ Firmicutes (<i>Staphylococcus</i> spp.)	Olivares et al. [17]
	↑ Gram (–) bacteria	Nadal et al. [19]
Duodenal mucosa	↓ Firmicutes (<i>Lactobacillus</i> spp., <i>Streptococcus</i> spp.)	Collado et al. [14]
	↓ Bacteroidetes (<i>Prevotella</i> spp.)	Schippa et al. [20]
	↑ Proteobacteria (<i>Neisseria</i> spp., <i>E. coli</i>)	Di Cagno et al. [21]
		Nistal et al. [22]
		Wacklin et al. [23]
Saliva	↓ Bacteroidetes	Sánchez et al. [24]
	↓ Fusobacteria	D’Argenio et al. [25]
	↑ Actinobacteria	Iaffaldano et al. [26]
Oropharyngeal swab	↓ Bacteroidetes	
	↓ Fusobacteria	
	↑ Actinobacteria (<i>Actinomyces</i> spp.)	
	↑ Proteobacteria (<i>Nf</i>)	

CD patients. They hypothesized that the higher levels of *Lactobacilli* they found in CD patients than in healthy individuals caused the higher CD-associated gluten-degrading activity observed in CD patients [27]. As shown

in Figure 1, we recently investigated the oropharyngeal and duodenal microbiomes of CD patients to evaluate if these two niches have some CD-associated microbial alterations in common [26]. Intriguingly, we found that the

Figure 1: Microbiome taxonomic composition in controls (C), in active (a-CD) and in GFD celiac patients.

The bar plots show the relative abundance (%) at phylum (A, B) and genus (C, D) levels in duodenal and oropharyngeal samples (A, C and B, D, respectively). Phyla and genera with an abundance greater than 1% in at least one study group are reported. Proteobacteria was the most abundant phylum in both duodenal and oropharyngeal samples of a-CD patients (A, B). The *Neisseria* genus was significantly more abundant in duodenum ($p < 0.005$) and in oropharynx ($p < 0.001$) of a-CD patients than in controls and GFD patients (C and D, respectively). The microbial profiles obtained in duodenal and oropharyngeal samples in each a-CD patient (E) had a very similar composition, indeed, the Proteobacteria phylum was most abundant phylum in duodenum and oropharynx niches (45% and 52%, respectively). The latter finding suggests a continuum from mouth to duodenum in the microbiome of a-CD patients (E). Error bars indicate standard error. Asterisks refer to taxa that differed significantly among three groups. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$. (Modified from Refs. [25] and [26] with permission.)

duodenum and oropharynx shared similar microbiome profiles, characterized by a high abundance of the Proteobacteria phylum, and *Neisseria* species, mostly accounting for this abundance. Our results suggest a continuum of microbiome in active CD patients from mouth to duodenum (Figure 1).

Duodenal microbiome

Interestingly, despite the different chemical and physical conditions present in the upper and lower tracts of the intestine, and different methodological approaches, some CD-associated fecal dysbiosis profiles were also observed in the duodenum of both children and adult CD patients compared to controls [13, 19–24]. In fact, there was an increased abundance of species belonging to the Proteobacteria phylum (*Escherichia coli* and *Neisseria*) and a decreased abundance of Firmicutes (*Lactobacillus*, *Streptococcus*) and Actinobacteria (*Bifidobacteria*) phyla [14, 19–24].

As shown in Figure 1, we recently characterized the duodenal microbiome profiles in active and GFD adult CD patients and controls using 16S rRNA sequencing, and, in agreement with above data, which were mostly obtained using diverse methodological approaches, found that Proteobacteria was the most abundant phylum, and Firmicutes and Actinobacteria were the least abundant phyla in the microbiome profiles of active CD patients [25, 26]. Furthermore, we highlighted that members of the *Neisseria* genus (β -Proteobacteria class), later identified as *Neisseria flavescens* (CD-*Nf*) by culture-based methods followed by mass spectrometry, were significantly more abundant in active CD patients than in GFD patients and control individuals [25, 26].

In conclusion, although some authors did not find any statistically significant difference between the duodenal microbiomes of CD patients and controls, probably due to the low resolution method used and/or to the low number of subjects studied [57–59], most of the scientific evidence is in line with a duodenal dysbiosis profile in patients affected by CD.

Fecal microbiome

Feces have been widely used to investigate gut CD dysbiosis in children. The early gut microbiota studies were based on culture methods combined or not with other technologies (denaturing gradient gel electrophoresis

[DGGE], fluorescence in situ hybridization [FISH], temporal temperature gradient gel electrophoresis [TTGE] and real time-polymerase chain reaction [RT-PCR]) and consequently did not identify all microbial taxa. Thanks to the advent of NGS, it is now possible to characterize all the bacteria in gut samples (Supplementary Figure 1). Most studies revealed an increase in Gram (–) vs. Gram(+) bacteria ratio, an increase in *E. coli* and *Bacteroides*, and a decrease in *Lactobacillus* and *Bifidobacteria* in feces from CD patients compared to controls [12–17] (Table 3). Furthermore, *Staphylococcus epidermidis* was more abundant in stools from CD children than in controls [14, 18, 24] (Table 3).

The putative pathogenic role of CD-associated dysbiosis

Numerous studies focused on the putative causal role of specific bacterial groups in the pathogenesis of CD [18, 25, 27, 54, 60–66]. Below, we summarize results that suggest the putative pathogenic role of specific microbes in CD.

Host immune-microbiota interaction

A higher abundance of virulent genes and *in vitro* production of pro-inflammatory cytokines was found in bacterial strains isolated from CD patients than in controls, as in the case of *Staphylococcus* spp. [18], *E. coli* [60], *Bacteroides fragilis* [61] and *N. flavescens* (*Nf*) [25]. In particular, using whole genome sequencing, we found that the gene system of iron acquisition differed between CD- and control-associated *Nf*. In fact, CD-*Nf* had the *hmbR* gene, which is usually present in the pathogenic *Neisseria* species *Neisseria meningitidis*, and lacked the *hpuA/B* and *tbpA/B* genes, present in commensal *Nf* strains [25]. Furthermore, CD-*Nf* induced inflammatory phenotypes in *ex-vivo* mucosal explants, and in both human and murine DCs [25] (Figure 2). We also observed statistically significant lower mitochondrial activity, accompanied by lower ATP production and by higher oxidative stress in CD-*Nf*-infected Caco-2 cells than in untreated Caco-2 cells [62]. Based on the aforementioned metabolic alterations, we hypothesized that the CD-*Nf* that we isolated from the duodenum of CD patients, could contribute to the pathogenesis of CD or to disease exacerbation.

In addition to bacteria, also viruses are involved in the pathogenesis of CD [54]. In fact, although reovirus infection does not cause symptoms it could interact with

Figure 2: *In vitro* inflammation effects of CD-associated *Nf*.

(A) *Nf* isolated from celiac disease (CD) patients induced a pro-inflammatory phenotype on dendritic cells. Filtered bacterial lysates from *Nf*, isolated from five active celiac patients (CD-*Nf*1, 2, 3, 7, and 8), promoted the production of pro-inflammatory cytokines by dendritic cells (DCs). CD11c+ DCs isolated from the spleens of C57B6 mice were cultured for 48 h in the presence of retinoic acid (RA) and transforming growth factor-β (TGF-β) with filtered *Nf* lysates from three active CD patients. All three tested bacterial lysates promoted the production of interleukin (IL)-12p40 by murine DCs, whereas untreated (* on horizontal bars), lipopolysaccharide (LPS)-treated (**), and LGG-treated DCs did not. (B) Human monocyte-derived DCs were cultured for 48 h in the presence of RA and TGF-β with filtered *Nf* lysates isolated from active CD patients. Tumor necrosis factor-α (TNF-α) levels were higher in supernatants of human DCs treated with *Nf*1, *Nf*7 and *Nf*8 bacterial strains isolated from small intestinal biopsies of CD patients, and in those challenged with *Nf*10 isolated from a pharyngeal swab of a control subject, than in those treated with bacterial LPS, used as a pro-inflammatory positive control (stars upon horizontal bars) and in untreated (UN) cells ($p < 0.0001$ for each of the treatments). IL-12p40 levels were higher in supernatants of human DCs challenged with *Nf*1, 2, 3 filtered lysates than in those treated with bacterial LPS (**) used as a proinflammatory positive control and in untreated (UN) cells (stars upon horizontal bars). Dot plots represent values from three independent experiments. Each experiment was performed in duplicate. A two-way analysis of variance was performed to check for inter-group differences. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. (C) Immunofluorescence analysis showed that in *ex-vivo* mucosal explants from control subjects the CD-*Nf* challenge enhanced the epithelial expression of the inflammation marker HLA-DR in villus enterocytes, in the basal cytoplasmic compartment as well as on the brush border and basolateral membranes. The HLA-DR scoring was graded from absent to very strong: 0–3 and measured the increase of HLA-DR expression. (Modified from Ref. [25] with permission.)

the immune system and affect its response to food antigens thereby promoting a pro-inflammatory rather than a regulatory response to them [54]. The latter finding was

supported by the break of tolerance to gluten in mice infected with reovirus and by antibody titers against reovirus in patients with CD vs. controls [54].

Gluten-microbiota interaction

As mentioned, the oral cavity is the first part of the gastrointestinal tract where wheat-derived gliadins may be substrates for oral microbial proteases originating from the bacteria naturally residing in the mouth [63]. In particular, *Rothia mucilaginosa* and *Rothia aeria* [64, 65] and *Lactobacilli* spp. [27], which are highly active towards gluten have been identified in the oral microbiota [27]. Other gluten-degrading organisms, including the immunogenic domains of gluten, are *Actinomyces odontolyticus*, *Streptococcus mitis*, *Neisseria mucosa* and *Capnocytophaga sputigena* [65]. Increased proteolytic activity against gluten has also been observed in the duodenum of CD patients in correlation with increased levels of Proteobacteria, including *Pseudomonas* [66]. Interestingly, in mice expressing CD risk genes, *Pseudomonas aeruginosa* elastase synergizes with gluten to induce more severe inflammation than that associated with mild villus blunting [66]. Moreover, microbial transglutaminase (mTG), an enzyme frequently used in food production, whose enzymatic properties are similar to those of human tissue transglutaminase (tTG), might be involved in the increase of CD [67]. In fact, mTG and gliadin are transported to the endoplasmic reticulum of enterocytes and mTG strongly localizes to the basolateral membrane and lamina propria, which indicates an antigenic interaction of mTG with cells of the immune system. Because mTG may also be released by bacteria (mainly those belonging to the Firmicutes phylum) within the intestinal microbiota, and also by external sources such as probiotics, mTG might play a role in the pathogenesis of CD [67, 68].

Concluding remarks

In conclusion, *in vivo* and *in vitro* studies definitively support an association between the gut microbiome and CD. Thus, both gluten-microbes and host-microbes interactions drive the gluten-mediated immune response. But, it remains to be established whether the CD-associated dysbiosis is the consequence of the disease, a simple concomitant association or a concurring causative factor. CD is a complex multifactorial disorder and there is no model system that summarizes its complexity. All current *in vitro* models, namely, human duodenal mucosal explants, immortalized cell lines (e.g. Caco2 cells), humanized mouse models, human intestinal organoids, have advantages and disadvantages (reviewed in ref. [69]). Recently, an intestine-on-a-chip system has been developed that forms polarized 3D-villus-like structures, and has basic

functional properties and an intestinal microenvironment [70]. This is the device that most closely resembles the *in vivo* human intestine and, hopefully, might help to clarify the role of the microbiome in the pathogenesis of CD. Indeed, by combining CD patient-derived human-induced pluripotent stem cells and human intestinal organoids, it will be possible to create intestine-on-a-chip systems that include individual's genetic background in order to implement also “personalized” therapeutic interventions.

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