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REVIEW

The double-edged sword of gut bacteria in celiac disease and implications for therapeutic potential

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Celiac disease (CeD) is an immune-mediated disease, triggered by gluten ingestion, in genetically susceptible individuals. The gluten-free diet (GFD) is the only current treatment for CeD, but is difficult to follow, has high non-adherence rates, and does not always lead to symptomatic or mucosal remission. Microbially-mediated mechanisms have been proposed to contribute to disease pathogenesis, and clinical studies support an association, but mechanistic insight has been difficult to obtain. Recent advances using translational approaches have provided clues to the mechanisms through which bacteria could contribute to CeD pathogenesis. In this review we discuss these bacterially mediated mechanisms, which include the modulation of pathogenic or protective pathways. Targeting these pathways through microbial therapeutics could provide adjuvant therapies to the GFD.

Celiac disease (CeD) is an autoimmune disease, triggered by the ingestion of gluten in genetically susceptible individuals, leading to inflammatory lesions in the proximal small intestine. The disease can develop at any age and presents with a spectrum of intestinal and extraintestinal symptoms and manifestations, the latter more common in patients with adult presentation. The presence of susceptibility genes, encoded by certain human leukocyte antigen (HLA) linked genes, and gluten consumption are necessary to trigger disease, but they are not sufficient. Over 90% of CeD patients carry the HLA-DQ2.5 haplotype, and the remaining are either HLA-DQ2.2 or HLA-DQ8, with a very small minority carrying the low-risk DQ7 haplotype.[1,2] Roughly 40% of the global population carry either DQ2 or DQ8, but only a fraction will go on to develop CeD.[3] Together with the fact that the prevalence of the disease is also on the rise,[4–6] suggests a critical role for additional genetic, and/or environment factors. The gut microbiota and its metabolites regulate many important immune functions, and as with other chronic immune-mediated diseases, are thought to be one of the key environmental triggers contributing to CeD onset or development.

Many of the key immunological pathways in CeD pathogenesis are well defined, making it a unique disease to study environmental triggers and co-factors. Tissue destruction in the small intestine (atrophy) results from complex interactions between gluten, genetics (HLA-DQ2/DQ8 molecules on antigen-presenting cells), the adaptive immune system (CD4 + T cells and B cells), and innate immune cells (intraepithelial lymphocytes; IELs). These concepts have recently been extensively reviewed.[7,8] Briefly, gluten peptides that are partially digested by host enzymes in the small intestine cross the epithelial barrier to the lamina propria where they are deamidated by tissue transglutaminase 2 (TG2). The deamidated gluten peptides bind with high affinity to HLA-DQ2 or DQ8 molecules on antigen-presenting cells, leading to the activation of IFN-γ-producing gluten-specific CD4 + T cells. The

gluten-specific CD4 + T cells are hypothesized to provide the help needed for both gluten-specific and TG2-specific B cell activation. In this hapten-carrier model, gluten-TG2 complexes are internalized by TG2-specific B cells and gluten is presented via HLA-DQ molecules to gluten-specific T cells, which in turn provide the help needed for B cell activation.[9,10] However, a gluten-specific T cell response is not sufficient for tissue destruction. This is highlighted in individuals with potential CeD, where anti-gliadin and anti-TG2 antibodies are present, but with normal small intestinal histology and IEL populations.[11] Small intestinal atrophy requires the activation and transformation of IELs to become cytotoxic cells, which mediate epithelial cell killing.[12] The production proinflammatory cytokines from T cells as well as expression of epithelial cell stress markers contribute to the activation of cytotoxic IELs,[13,14] but other unknown triggers are thought to participate.

Clinical studies support the hypothesis that changes in gut microbiota composition, including the virome, impact CeD risk. However, inferring precise mechanisms from clinical studies is challenging, and universal interpretation of results is difficult due to heterogeneity in design, techniques, disease status, and most importantly, sampling location.[7] While prospective longitudinal studies in genetically susceptible children have provided key insight into the "pre-disease" microbial state,[15–17] the identification of microbial-mediated mechanisms that drive disease, or protect from disease development, are still unclear. Translational approaches are needed to better define underlying mechanisms (Fig. 1).

In this review we will focus on the translational and mechanistic studies that have investigated the role of the microbiome on host reactions to gluten. We will discuss new advances that provide insight into microbially-mediated pathways, and the gaps in knowledge that could guide future research. We will specifically describe bacterially-mediated mechanisms, acknowledging that certain viruses capable of inducing an inflammatory host response

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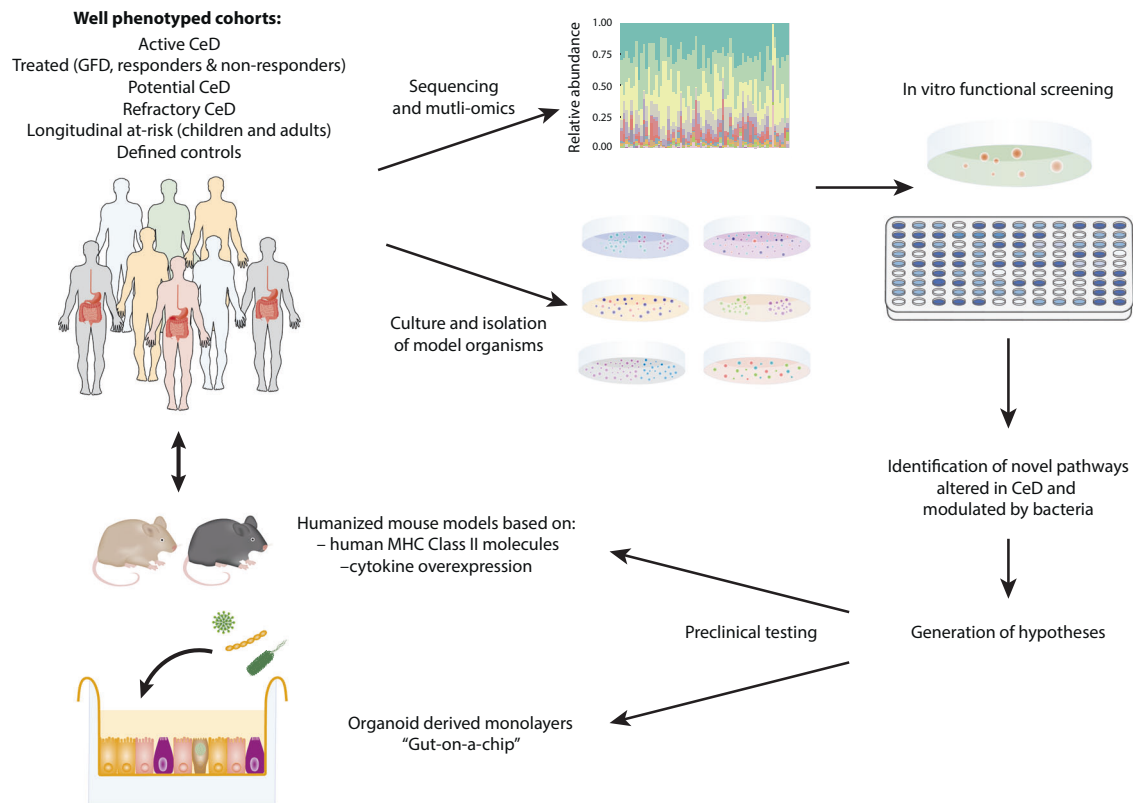
Fig. 1 Pipeline for translational bench to bedside approach to study microbiome role in celiac disease (CeD). Clinical samples (feces, duodenal biopsies, or small intestinal aspirates) from well phenotyped and characterized cohorts of CeD patients and control populations can be sequenced for microbiota composition and functional analysis, and cultured for the isolation of model organisms. In vitro assays can be used to screen model organisms or clinical samples for specific functions, leading to the identification of novel microbial-mediated pathways in CeD and the generation of hypotheses, which can be tested using relevant in vitro (organoid-derived monolayers, gut-on-a-chip) and mouse models (expressing human CeD risk gene or key cytokines, such as IL-15).

play a key role in CeD,[18,19] a topic that has been extensively reviewed recently.[7,20] Clinical data supporting the link between altered microbiome profiles in celiac patients has also been extensively reviewed elsewhere[7] and will not be the focus of this review.

EARLY BASIC STUDIES EXPLORING A MICROBIOME ROLE IN GLUTEN IMMUNOPATHOLOGY
More than two decades ago, and prior to the first clinical descriptions of altered microbiota composition in CeD, the potential role of the gut microbiota on host responses related to gluten was addressed in a study using axenic rodents.[21] This study showed that germ-free rats, but not conventionalized rats that were fed wheat gluten from birth, developed intraepithelial lymphocytosis. Germ-free rats fed albumin at similar doses did not develop increased IELs, suggesting some specificity to the antigen challenge. The study did not discriminate between responses to gluten, or non-gluten proteins complexed with crude gluten, such as amylase trypsin inhibitors (ATI). Nevertheless, it raised the hypothesis that in the absence of microbes, responses to immunogenic wheat proteins can develop. Similar results were reported in a subsequent study using mice expressing the CeD genetic susceptibility gene, DQ8, on a non-obese diabetic (NOD) background, which allows for recognition of gluten epitopes by MHC-class II. Adult germ-free NOD-DQ8 that were sensitized to gliadin using the mucosal adjuvant cholera toxin, followed by gluten challenge, developed more severe decreases in villus to crypt ratios than mice harboring an ultra-clean microbiota, free from any pathogens.[22] The results suggested that in the absence

of microbial immune regulation, host responses to gluten are more inflammatory. The study indicated that microbiota composition also dictated the degree of gluten immunopathology that developed in the model. NOD-DQ8 mice colonized with the ultra-clean microbiota Altered Schaedler flora (ASF) were protected from gluten-immunopathology, whereas mice colonized with a conventional specific pathogen-free microbiota developed moderate enteropathy. Gluten immunopathology in adult sensitized mice was also exacerbated if mice were perinatally treated with vancomycin, which led to increased abundance of Proteobacteria, including *Escherichia*. Similarly, when ultra-clean mice were colonized with an enteroadherent strain of *Escherichia coli*, isolated from a celiac patient, mice developed more severe gluten immunopathology following sensitization in adulthood.[22] The studies shed light on the potential role of microbial factors in CeD pathogenesis, which include protective as well as harmful effects, although the molecular mechanisms remained elusive. Early in vitro studies also support this 'double-edged sword' of the microbiota in host responses to gluten. *E. coli* or *Shigella*, isolated from a CeD patient, led to inflammatory responses, characterized by IFN-γ and IL-12 production, following stimulation of peripheral blood mononuclear cells (PBMCs)[23] or dendritic cells (DCs).[24] On the other hand, stimulation of PBMCs or DCs with bifidobacteria led to the production of the regulatory cytokine IL-10 in the presence of gliadin. *E. coli* and *Shigella*, but not bifidobacteria, also induced DC maturation.[24] Similarly, *E. coli* and *Shigella* induced altered tight junction ZO-1 expression in intestinal loops in germ-free rats, leading to gliadin peptide translocation to the lamina propria. *Bifidobacterium bifidum*, on the other hand, reversed gliadin-induced alterations in ZO-1 expression.[25] The implications
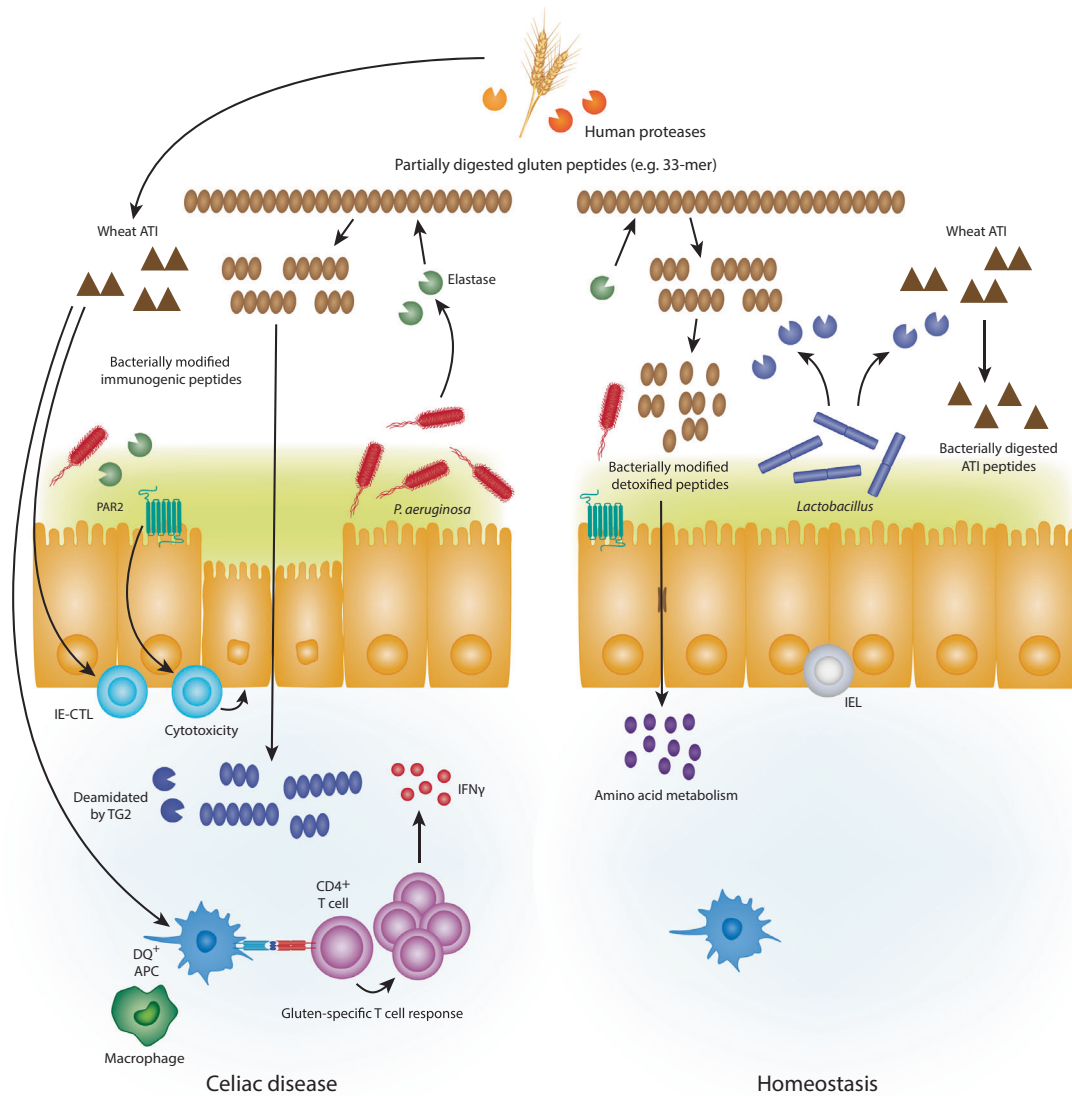
Fig. 2 Celiac disease (CeD) mechanisms mediated through microbial elastase. Gluten peptides can be digested by bacterial proteases (i.e., elastase from *P. aeruginosa*) to produce immunogenic gliadin peptides that can easily translocate the small intestinal epithelial barrier and stimulate proinflammatory T cell responses in CeD patients. Other bacteria, (i.e., *Lactobacillus* spp.) can further degrade immunogenic gliadin peptides as well as other inflammatory wheat proteins like amylase trypsin inhibitors (ATI), resulting in detoxification. At the same time, microbial proteases (i.e., elastase from *P. aeruginosa*) can also stimulate intraepithelial lymphocytes (IELs) through protease activated receptor-2 (PAR-2) cleavage to become cytotoxic IELs (IE-CTL). APC, antigen-presenting cell; TG2, tissue transglutaminase 2.

and significance of these in vitro bacterially-mediated responses in vivo, in a genetically susceptible host colonized with a complex community of microbes and exposed to gluten, remains unclear.

The search for a mechanism using translational and multidisciplinary approach

Elucidating specific microbially-mediated mechanisms is key for the development of new microbiome-based therapies, an area of growing interest in gastroenterology and medicine.[26] Reaching this goal will require translational and multidisciplinary approaches, where clinical questions are tested in disease relevant in vivo and in vitro models, and then confirmed in well-phenotyped clinical cohorts (Fig. 1). One advantage that CeD has over other autoimmune and inflammatory diseases, like type 1 diabetes or inflammatory bowel disease (IBD), is that the major susceptibility genes (DQ2 and DQ8), the main environmental trigger (gluten), and the autoantigen (TG2), are all well defined. This means reactivation and remission of disease can be

manipulated in experimental settings and the inflammatory events following gluten ingestion can be detected with specific biomarkers, including CeD specific serology,[27,28] and new biomarkers, such as the early rise in IL-2 after gluten challenge.[29] Moreover, one key milestone achieved in the past decade in CeD is the development of a variety of animal models based on human MHC-class II expression, that can be manipulated to reproduce relevant pathways of disease.[30] Advancement of sequencing techniques and deep culture capacity will continue to facilitate the identification of "model microbial organisms" from well-phenotyped patients, that can be tested in these models to understand molecular mechanisms (Fig. 1). The use of human microbial communities and or clinical isolates (model organisms) in gnotobiotic mouse models, that are also humanized from an immune perspective (i.e., expression of human MHC Class II, or cytokines of interest),[14,22,31] greatly enhances the translational relevance. Although limited to the study of single isolates, rather than community dynamics, this approach based on "model
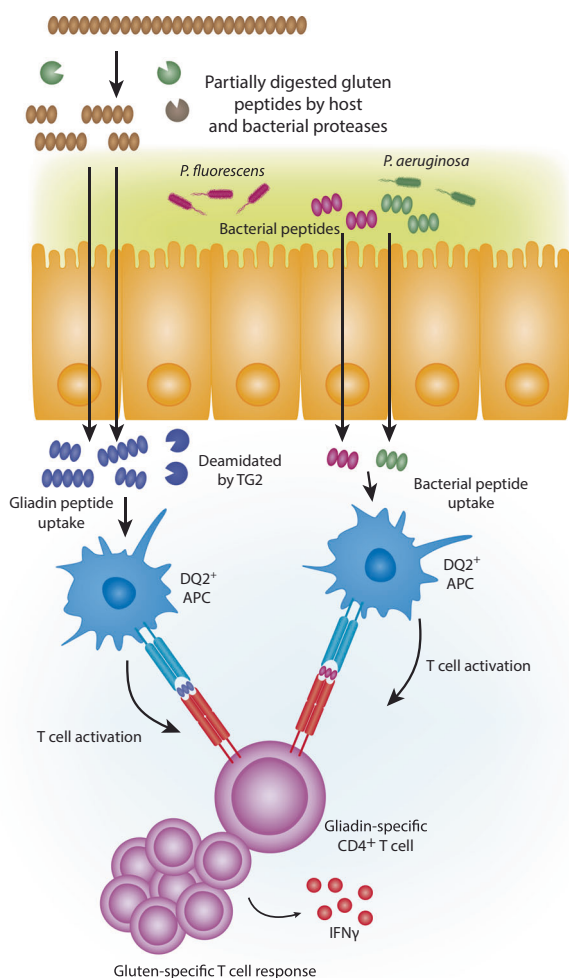
Fig. 3 Microbe-gluten molecular mimicry in celiac disease (CeD). Microbially derived peptides (i.e., from *P. fluorescens* or *P. aeruginosa*) that mimic immunogenic gliadin peptides can be processed and presented by antigen-presenting cells (APC) to activate T cells from patients with CeD. TG2, tissue transglutaminase 2.

organisms" has been undertaken to explore the important role of pathogenic viruses in CeD.[18,19] The following sections will discuss specific gluten-bacterial-host interactions discovered based on the use of model bacterial organisms or microbial communities.

Pseudomonas, a taxon mediating specific pathogenic mechanisms in CeD

While several bacterial taxa have been implicated in CeD pathogenesis, the *Pseudomonas* genus has been one of the most well studied in terms of molecular mechanisms linked to CeD pathogenesis. *Pseudomonas* was first recognized as a potential taxa of interest in CeD when it was discovered that, similarly to IBD, patients with active CeD have higher levels of serum antibodies towards *Pseudomonas fluorescens*-associated sequence (anti-I2).[32] Anti-I2 antibodies were also detected during early stages of the disease,[33] and remained elevated in those poorly responsive to the gluten-free diet (GFD).[34,35] This suggests that immunoreactivity to microbial antigens from rare, but potentially pathogenic species[36] could play a role in CeD development or perpetuating inflammation during active disease. However, no mechanistic insight emerged from these studies. Other species in the *Pseudomonas* genus, like *P. aeruginosa*, have also raised interest for their potential role in CeD. *P. aeruginosa* is an opportunistic pathogen that is a major cause of severe infections

among hospitalized individuals and those that are immunocompromised. *P. aeruginosa* frequently causes chronic lung infections in patients with cystic fibrosis (CF);[37] however, in healthy individuals, exposure to *P. aeruginosa* usually does not lead to lung or gastrointestinal infections, even though humans are frequently exposed to it in a variety of terrestrial and aquatic environments, and it can be isolated from stool samples of healthy individuals.[38] It is difficult to conclude whether detection of *Pseudomonas* in the gut indicates passage or colonization of the gastrointestinal (GI) tract, but opportunistic pathogen presence may be sufficient to induce host responses, in a genetically predisposed host or an individual with pre-existent proinflammatory changes. Interestingly there is an increased prevalence of CeD among CF patients, and it is possible chronic *P. aeruginosa* colonization in CF patients could contribute to this increased risk.[39,40]

Mechanistic interest in *P. aeruginosa* in the context of CeD grew after being isolated from the stool of healthy individuals[38], as well as from the small intestine of celiacs[41] and in vitro functional studies demonstrated its ability to metabolize gluten.[38,41,42] Digestive proteolytic resistance of gluten is indeed one of the main determinants of its immunogenicity, as this will lead to gluten peptide transport across intestinal epithelial cells,[43] interactions with antigen presenting cells, and antigen presentation to T cells in genetically predisposed individuals. Initially, it was hypothesized this efficient in vitro gluten metabolism by *P. aeruginosa* could be exploited for therapeutic purposes, by identifying the enzymes involved in this process.[38] However, a subsequent study demonstrated potential pathogenic effects of *P. aeruginosa*-digested peptides (Fig. 2).[42] The authors first used a gnotobiotic approach to demonstrate that different bacteria, such as *P. aeruginosa* and *Lactobacillus* spp. isolated from a celiac patient or healthy control, produce distinct gluten-degradation patterns in the small intestine. This was supported using in vitro assays where the authors tested the ability of these isolates to digest immunogenic gliadin peptides, including the 33-mer. They found that while *P. aeruginosa* was able to cleave the 33-mer, leaving a variety of smaller peptides that were 10-30 amino acid in length, it was not able to cleave QLP regions in the 33-mer, which are associated with high immunogenicity.[44] Using a genomic approach with a non-redundant transposon mutant library of the clinical *P. aeruginosa* PA14 isolate, it was found that the major enzyme involved in this gluten metabolism was LasB, or elastase B, one of its major virulence factors.[42] The resulting peptides from bacterial digestion were also identified through liquid chromatography tandem mass spectrometry (LC-MS/MS). Those generated from *P. aeruginosa* degradation better stimulated IFN-γ-producing gliadin-specific T cells from celiac patients compared to the 33-mer, and were better able to translocate across the epithelial barrier compared to the 33-mer. Interestingly, the authors found that *Lactobacillus* spp., which was isolated from a healthy subject, was not able to efficiently degrade the 33-mer peptide; however, *Lactobacillus* spp. could detoxify some of the *P. aeruginosa*-produced peptides.[42] These studies suggest that gluten digestion in vivo is a sequential and complex event that includes both host and microbial proteases, and the type of microbial proteases could determine immunogenicity of gliadin peptides that are present in the small intestine.

In summary, translational studies using relevant in vitro and in vivo animal models, as well as "model" clinical bacterial isolates, have shown that gluten metabolism is a complex event that involves host digestive enzymes[45] and the small intestinal microbial community.[7] Many bacterial taxa from the oral cavity, small intestine, and feces are capable of degrading gluten and the immunogenic gluten peptide, 33-mer.[41,46–50] However, the collective gluten metabolic capacity of the microbiome along the gastrointestinal tract is individual.[41] What remains to be determined are the factors that influence this activity and whether

they could be manipulated to promote optimal endogenous gluten detoxification, without promoting inflammatory pathways.

In addition to this gluten metabolism mechanism, direct inflammatory effects of elastase from *P. aeruginosa* could contribute to inflammation in CeD (Fig. 2). In a subsequent study by the same group, the authors confirmed that small intestinal biopsies from celiac patients had higher proteolytic activity towards gluten, suggesting an increase in bacteria that can use gluten as an energy source, compared with healthy controls. Moreover, this correlated with the abundance of microbes with known gluten-degrading capacity, such as *Pseudomonas*.[51] The authors then colonized germ-free mice with *P. aeruginosa*, or a mutant of *P. aeruginosa* that lacked elastase activity (LasB). Bacterial elastase induced inflammation and increased IELs in the small intestine of wild-type mice through a gluten-independent mechanism that involved the protease-activated receptor-2 (PAR-2) pathway, as no effect was seen in protease-resistant PAR-2 mutant (R38E-PAR2) mice.[52] However, *P. aeruginosa* elastase synergized with gluten to induce more severe inflammation and moderate small intestinal villus blunting in mice expressing CeD risk genes (HLA-DQ8).[51] Together these studies point to both gluten-specific and non-specific effects that could influence key pathways in CeD, mediated through bacterial (*Pseudomonas*) elastase (Fig. 2). Moreover, transfer of CeD duodenal microbiota to germ-free wild-type mice also led to non-specific inflammation, including increased IELs, suggesting many taxa could be involved in similar mechanisms.

Another study proposed a third mechanism for the *Pseudomonas* genus in CeD, independent of gluten metabolism or bacterial elastase. Given the described serological reactivity to *P. fluorescens* in active CeD, Petersen et al. discovered that DQ2.5-restricted gliadin epitopes had a high similarity to peptides from *P. fluorescens* and *P. aeruginosa* (Fig. 3). The bacterial peptides cross-reacted to gliadin-specific T cells, either from cell lines transduced with TCRs specific for DQ2.5 specific gliadin peptides, or from celiac patients.[53] This study suggests that molecular mimicry between gliadin and microbial peptides can occur and this mechanism could drive, initiate, or maintain disease. Whether these microbial peptides cross the small intestinal barrier and are presented to T cells via DQ2.5 in vivo has yet to be tested. Cross-reactivity could occur between other bacterial or viral peptides and gliadin, but this has yet to be discovered.

Proteobacteria: a common phylum of interest in CeD?
In addition to *Pseudomonas*, other groups from the Proteobacteria phylum have been implicated for their possible role in CeD pathogenesis. Clinical studies have shown that Proteobacteria are overrepresented in the duodenal and oropharyngeal mucosal of adults with CeD.[54–56] Specifically, the abundance of *Neisseria* has been shown to be significantly higher in active CeD patients. *Neisseria flavescens*, an opportunistic pathogen, was the most abundant *Neisseria* species in the duodenum of active celiacs but was absent in the duodenum of controls. Additional studies demonstrated that *N. flavescens* strains isolated from the duodenum of patients with CeD expressed genes associated with virulence, and induced an inflammatory response in DCs and mucosal explants in vitro.[54] These results need confirmation in additional clinical cohorts, and also translational studies to confirm mechanisms relevant in CeD pathogenesis. Another study linked increased abundance of Proteobacteria to persistent symptoms in patients with CeD on a GFD, but species and mechanisms involved remained unknown.[57] *Escherichia coli* has frequently been found to be increased in the duodenum of patients with CeD, particularly in children.[58,59] Indeed, *E. coli* isolated from CeD patients had increased carriage of virulence genes compared to strains isolated from controls.[60] Similar to the in vitro effects of *N. flavescens*, *E. coli* strains isolated from CeD patients induced an inflammatory response in PBMCs, DCs, and intestinal loops.[23–25] Moreover, an enteroadherent strain of *E. coli* from patient with CeD was found to increase gluten-immunopathology in mice expressing the DQ8 celiac risk gene.[22] The exact mechanisms through which *E. coli* may contribute to CeD pathogenesis is unclear, but could be related to increased virulence and mucosal adhesion promoting an inflammatory milieu.

The double edged-sword of the microbiota: Are there potential protective taxa in CeD?
In addition to the pathogenic potential of bacteria in CeD, protective microbially-mediated mechanisms have been described in other chronic inflammatory conditions such as inflammatory bowel disease.[61] By understanding proinflammatory events mediated by bacterial opportunistic pathogens, insight into protective mechanisms may arise. Like *P. aeruginosa*, many other bacteria that naturally colonize or are found in the GI tract have gluten-degrading capacity.[41] However, it is important to determine the immunogenicity of the resulting peptides following metabolism. While some taxa can degrade gluten leading to more immunogenic peptides, as was described for *P. aeruginosa* above, others are able to detoxify gluten peptides, or non-gluten immunogenic proteins that can have adjuvant proinflammatory effects in CeD,[62,63] so they are no longer immunogenic (Fig. 2). *Lactobacillus rhamnosus* X-32.2 and *L. fermentum* X-39.3 isolated from a healthy control subject were shown to detoxify the immunogenic gliadin peptides that resulted from *P. aeruginosa*-mediated digestion. Unlike the 33-mer or *P. aeruginosa*-produced peptides, the *Lactobacillus*-produced peptides had lower immunogenic capacity when incubated with gliadin-specific T cells isolated from CeD patients.[42] It is important to note that in these in vitro experiments, *Lactobacillus* was not able to initiate 33-mer digestion, but required initial digestion by *P. aeruginosa* elastase. Gluten-degrading bacteria have also been isolated from the oral cavity.[46,48] *Rothia mucilaginosa* is a natural colonizer of the oral cavity that is associated with oral health, and was able to degrade several immunogenic epitopes on the 33-mer in vitro.[64] Similarly, *Rothia aeria* could degrade several immunogenic gliadin peptides in vivo.[65] While promising, the immunogenicity of peptides resulting from *Rothia* degradation should be confirmed using T cell stimulation assays, and relevant animal models, before using these strains in clinical trials. Overall, these results emphasize the complexity of microbiota metabolism of proteolytic resistant proteins such as gluten, which need to be taken into account when developing enzymatic therapies aiming at gluten detoxification.

Certain strains of lactobacilli also have the capacity to degrade and detoxify other non-gluten wheat proteins implicated in CeD (Fig. 2).[62] ATIs are also poorly digested in the GI tract, and were shown to induce an innate immune response via toll-like receptor-4.[63] A subsequent study confirmed that ATIs induced gut dysfunction and increased IELs, through both MyD88/TICAM and IL-15 pathways.[62] This ATI-induced innate immune activation exacerbated gluten immunopathology in DQ8-expressing mice, and was associated with an altered microbiota composition, including decreased abundance of *Lactobacillus*. Using a library of bacteria isolated from the GI tract of healthy controls, the authors screened for bacteria with ATI-degrading capacity. Of note, several strains of *Lactobacillus* showed high capacity to degrade ATI, and when supplemented to mice could reduce ATI-induced immune activation and the exacerbation of gluten-immunopathology. Other ATI-degrading bacteria were identified, but the protective capacity using relevant animal models has yet to be tested.[62]

Another protective bacterially-mediated mechanism in CeD relates to the aryl hydrocarbon receptor (AhR) pathway (Fig. 4). Like in IBD and metabolic disease,[66,67] the microbiota of patients with active CeD was shown to have impaired capacity to metabolize tryptophan to produce ligands (indoles) of the immunoregulatory and barrier protecting AhR pathway.[68] The
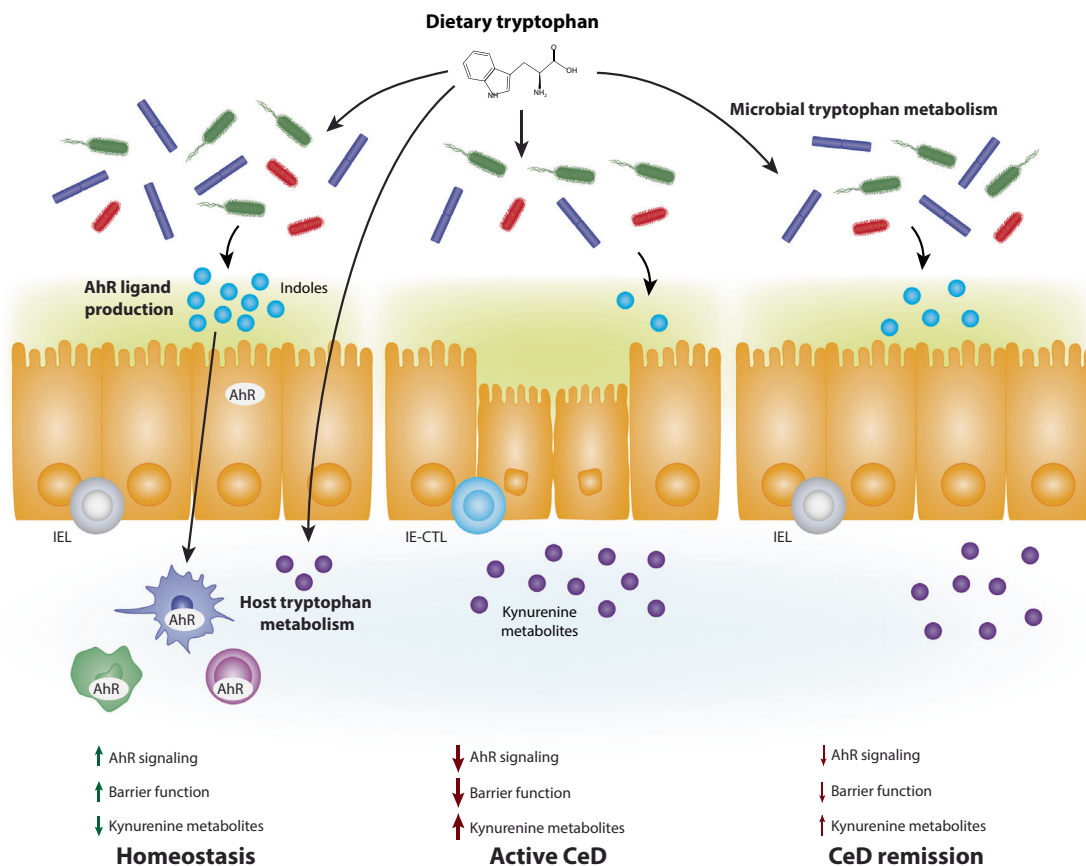
Fig. 4 Microbial tryptophan metabolism in celiac disease (CeD). In healthy individuals, tryptophan in food is metabolized by gut microbes into indoles, and derivatives that can activate the aryl hydrocarbon receptor (AhR). Conversely, the microbiota in those with active CeD has impaired capacity to metabolize tryptophan to AhR ligands, and tryptophan metabolism is shifted to the kynurenine pathway, which is implicated in inflammatory diseases. The impaired tryptophan metabolism is partially restored in CeD patients after 2-years on a gluten-free diet (GFD), resulting in reduced kynurenine metabolites and increased microbiota-dependent AhR ligands. IEL, intraepithelial lymphocyte; IE-CTL, cytotoxic IEL.

authors found that this is only partially restored two years after a GFD.[68] To further study whether this pathway could be targeted with microbiota-modulating therapies, the authors supplemented DQ8-expressing mice with lactobacilli strains known to produce AhR ligands (*L. reuteri* CNCM-I5022 and *L. reuteri* CNCM-I5429).[66,67] They found that *L. reuteri* treated mice had increased AhR activity in the small intestine, and improved gluten-immunopathology. Supplementing the mice with a high tryptophan diet modulated gut microbiota composition, leading to increased abundance of AhR ligand producers such as *Lactobacillus* and *Ruminococcus gnavus*. The high tryptophan diet also upregulated AhR ligand production and reduced gluten immunopathology in DQ8-expressing mice.[68] This study identifies a potential pathogenic mechanism in CeD, that could be targeted through specific probiotic treatment, or dietary approaches to modulate AhR ligand production by the intestinal microbiota. Not all lactobacilli tested demonstrated activation of AhR pathway, highlighting the importance of characterizing specificity and strain effects before therapeutic intervention.

Gut proteolytic imbalance has been described in many chronic inflammatory conditions,[69–71] and recently a bacterial component that contributes to this imbalance was described in IBD.[72] Proteolytic activity in the GI tract is tightly controlled by a system of anti-proteases, to prevent dysregulated intestinal inflammation and damage. Anti-proteases are produced by both host cells[73] and gut bacteria[74,75] and this opens another potential pathway for protective taxa in CeD. Indeed, the expression of elafin, a human serine protease inhibitor, was decreased in the duodenum of patients with active CeD. Delivery of elafin to the small intestine of DQ8-expressing mice using a recombinant *Lactococcus lactis* engineered to produce elafin, prevented gluten-induced immunopathology.[76] Bacteria, including the infant-derived probiotic *Bifidobacterium longum* NCC2705, naturally produce serine anti-proteases (serpins). Given the concerns and clinical application of recombinant, genetically modified bacteria, a follow-up study demonstrated that serpin from *B. longum* NCC2705 could protect DQ8-expressing mice from gluten-immunopathology.[77] *B. longum* CECT 7347 was shown to have potentially beneficial effects in a rat model of gliadin-induced enteropathy, but mechanisms were not explored and whether this is related to serpin activity or other anti-inflammatory properties is unknown.[78]

Other microbial metabolites, such as short chain fatty acids (SCFA) that are derived from microbial fiber fermentation, have immune and barrier modulating effects that are relevant in CeD pathogenesis.[20] Butyrate, for example, is an important modulator of regulatory T cells, which play an important role in oral tolerance to food antigens.[79] In addition, butyrate[80] and acetate[81] have barrier promoting effects, and butyrate was shown to reduce gliadin-induced IFN-γ and IL-15 production from organoid-derived monolayers derived from CeD biopsies.[81] Interestingly, over-expression of IL-15 in the mouse epithelium led to a reduction in butyrate producing-bacterial taxa, which increased susceptibility to colonic inflammation.[82] It is unknown whether the high levels of IL-15 that are observed in some CeD patients leads to reduced butyrate, and whether this contributes to CeD onset or progression. While alterations in SCFAs and SCFA-producing

bacteria have been reported between CeD subjects and healthy controls,[83,84] more mechanistic studies are needed to confirm the protective effect, if any, of SCFAs in CeD pathogenesis.

The potential protective mechanisms mediated by the microbiota are only beginning to emerge in CeD. Indeed, as we learn more about functional roles of the bacteria and other non-bacterial components of the microbiota from other inflammatory diseases like IBD or food allergy, we can study their potential roles in CeD. For example, whether certain microbes play a role in preventing the breakdown of oral tolerance to gluten would be key for exploring preventative measures in populations at-risk for CeD.

Rationale and future perspective on microbiota modulating therapies in CeD

The only treatment for CeD is a strict gluten-free diet (GFD), which is difficult to follow and expensive, resulting in high non-adherence rates. Accidental contamination is common and very small amounts of gluten (~50 mg) can cause inflammation.[85–88] Mucosal recovery after starting a GFD is slow, and more than 60% of patients have persistent mucosal inflammation even after 5 years of a GFD.[89] This is clinically important because long-term, low-grade mucosal injury increases bone fracture risk and predisposes to nutritional deficiencies. In addition, despite following a GFD, up to 30% of CeD patients will have persistent symptoms.[87,90,91] The requirement for continuous monitoring of food intake has a negative impact on patients' quality of life, and there is an unmet need for adjuvant therapies.[92] In addition to taxa that are potentially harmful in CeD, evidence suggests that several taxa may be beneficial, and microbiota-modulating strategies have been suggested to supplement the GFD as a therapeutic strategy.

A number of different probiotics have been tested in CeD; however, the conventional probiotics tested until now have not provided any concrete evidence to support the use of probiotics for CeD. In line with this, a recent meta-analysis concluded that probiotics may improve gastrointestinal symptoms in those with CeD, but higher quality evidence is needed before any recommendations can be made due to the heterogeneity between studies.[93] The probiotics tested were selected with no specificity for CeD, but based on their overall claimed anti-inflammatory properties. Future probiotics or microbial-based therapies should focus on microbes that target specific pathways relevant for CeD. For example, targeting wheat protein digestion is an exciting and promising approach, which would target gluten or other inflammatory wheat proteins before they can induce an inflammatory immune response in the body. Other promising microbial therapeutic targets for CeD include the restoration of AhR signaling, through microbial tryptophan metabolism, or of intestinal proteolytic balance, through serine protease inhibitors.

An important concept to consider however, is that not all probiotics are equal. Not all strains of lactobacilli can produce AhR ligands or can degrade immunogenic gluten peptides. Similarly, not all strains of bifidobacteria can produce anti-proteases, and not all anti-proteases will target the same protease. Testing potential strains in relevant animal models or in vitro settings to confirm functional properties is essential before moving to clinical trials, to enhance translational success. Combination therapies where multiple probiotics, or a probiotic that targets multiple pathways could also be explored, but these should follow a rational mechanistic-based approach. The use of engineered bacteria also deserves discussion. Next-generation microbial therapeutics constitutes a new category of drugs and includes live biotherapeutic products (LBP) and genetically modified microorganisms, engineered to produce or express genes of interest relevant to CeD pathogenesis. Engineered bacteria have been tested in animal models of CeD, but their use in humans is limited due to the use of plasmids.[76] Novel methods where genes of interest are integrated into the bacterial chromosome of currently available probiotics, could limit horizontal gene transfer and dissemination. Finally, the role of fungi[94] and protists[95] is emerging in many chronic GI diseases, such as inflammatory bowel disease, but has been unexplored in CeD. It is expected to yield a new field of study in the future, which in turn could help us develop better targeted therapies.

These novel therapies could be used both in CeD patients that are non-responsive to the GFD to help control ongoing symptoms and aid in mucosal recovery, and in at-risk populations in a preventative approach. Along these lines, longitudinal studies can help guide proof-of-concept translational studies, where samples from "pre-disease" state can interrogate the role of microbes and mechanisms involved in initiation of disease.[72] However, moving from clinical samples, to mechanistic studies, back to proof-of-principle clinical trials requires significant investment, which traditionally has been lacking in CeD. Thankfully, in recent years there has been a surge of interest in CeD driven by both the public and industry which has the potential to drive research into a new age. We have still a long way to go before the application of biotherapeutics in CeD in clinical practice, but as the spotlight on this condition brightens, the potential for effective preventative measures and effective treatments for symptoms becomes a clearer reality.

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