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The role of microbiome in the development of gluten-related disorders

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ABSTRACT

Gluten-related disorders (GRD) include celiac disease (CD), non celiac gluten sensitivity (NCGS) and wheat allergy (WA), conditions that are associated with the ingestion of gluten-containing food. Gut microbiota composition and function may be involved in the pathogenesis of GRD. In untreated CD the microbiota is characterized by a reduction in beneficial microbes like *Lactobacillus* and *Bifidobacterium* and an increase in pathogenic ones such as *Bacteroides* and *E. coli*. Dysbiosis is a hallmark of CD, persists across various disease stages and is only partially corrected by a gluten-free diet. NCGS patients show a different microbial profile, with a notable decrease in microbial richness, and an increase of Ruminococcaceae and decrease of *Bacteroidetes* and *Fusobacteria*. The increase of certain bacterial groups such as *Clostridium* and *Anaerobacter*, in contrast with the decline of *Bacteroides* and *Clostridium XVIII*, marks a distinctive microbial signature associated with allergic responses to food. Mechanisms linking the gut microbiota to the development of GRD include effects on the gut barrier function, microbiota-mediated immune response to gluten, and an impact of microbial metabolites on gluten digestion and tolerance.

Although the gluten-free diet is the primary therapy of GRDs, treatment with probiotics may contribute to improve the natural history of these disorders, for instance by minimizing the damaging effects of gluten contamination and accelerating the catch-up growth at the beginning of the dietary treatment of CD. Additional high-quality trials are still needed to identify and standardize the use of probiotics/prebiotics in GRDs.

1. Introduction

"Gluten related disorders" (GRD) is an umbrella term including a group of conditions associated with the ingestion of food containing gluten [1],a protein complex that is found in wheat and other grains (barley and rye) and their derivatives [2]. Celiac disease (CD), non-celiac gluten sensitivity (NCGS), and wheat allergy (WA) are the most important disorders linked to gluten consumption. They are characterized by an aberrant immune response after exposure to wheat and other aforementioned grains, potentially leading to clinical manifestation of disease [3]. Specifically, gluten ingestion provokes a T-cell mediated response and production of anti-tissue transglutaminase and anti-endomysial antibodies in individuals with CD. Consequently, the small intestinal mucosa undergoes histologically characteristic alterations, featuring villous atrophy and crypt hypertrophy [4]. Patients present with intestinal and extraintestinal symptoms like diarrhea, malabsorption, and fatigue, however, many remain symptomless. CD can emerge at any point in time, in subjects ingesting gluten and showing genetic predisposition, i.e. positivity of HLA-DQ2 and/or HLA-DQ8 genes [3]. WA depends on a different type of mechanism, namely a T helper 2 inflammatory reaction, Immunoglobulin E (IgE)--mediated. Individuals affected by IgE-mediated WA develop symptoms such as asthma, diarrhea and vomiting. WA occurs within a brief time window, typically spanning from minutes to hours from gluten ingestion/inhalation [5]. Lastly, NCGS is characterized by the presence of intestinal and extraintestinal symptoms which manifest a few hours to

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days following the consumption of grains containing gluten. Clinical presentation closely parallels that of CD, nevertheless NCGS does not conform to the diagnostic criteria of either CD or WA [6]. All the previously mentioned GRD can be treated with the implementation of a gluten-free diet [3,7]. Wheat, a staple food in human nutrition, stands as an ingredient in many baked and processed food items consumed worldwide [2]. It contributes to approximately 20 % of the total global caloric and protein intake [8]. The increasing adoption of Westernized dietary patterns is responsible for a rise in the incidence of GRD, currently estimated around 3–4% of the general population in most countries, thus establishing an emerging health concern [1,3].

Over the last few decades, there has been a growing evidence of the potential involvement of the microbiota in the initiation and progression of GRD. In the broader context, gut microbiome has a great direct and indirect impact on the host's state of health. Its composition can change according to the presence and progression of a disease. Under normal physiological conditions, the bacterial component of the microbiota is constituted by 7 main entities: *Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria, Verrucomicrobia* and *Cyanobacteria*. Microorganisms residing in the gut maintain a commensal symbiosis with the host intestinal environment. They show important functions such as breaking down fibers indigestible by the host, operating alongside the immune system in maintaining gut barrier, managing the permeability of the intestinal wall, and communicating with the Central Nervous System through the gut-brain axis [9,10].

Several environmental factors, including gut microbiota composition and function, may be involved in the pathogenesis of GRD [11]. In the case of CD, genetic predisposition is a necessary but not sufficient factor to develop the disease. Indeed, 40 % of individuals of Caucasian descent carry the HLA-DQ2/DQ8 haplotype, but only a small fraction of them will develop CD. Hence, the onset of CD requires involvement of other contributing elements like the gut microbiota [3]. Another important aspect emphasizing the potential role of microbiome in pathological processes underlying CD pertains to patients with refractory forms of the disease, who exhibit unresolved dysbiosis despite adhering to a gluten-free diet [7].

This review article focuses on the interindividual variability of gut microbial composition and its impact on the pathogenesis of GRD. Additionally, possible applications of therapeutic strategies based on the modulation of gut microbiota in patients with such conditions will be discussed.

2. Microbiota composition in individuals with GRD

2.1. Microbiota changes in individuals with CD, NCGS and WA

In untreated CD, the microbiota is markedly unbalanced, a condition known as dysbiosis, with a reduction in beneficial microbes like *Lactobacillus* and *Bifidobacterium* and an increase in pathogenic ones such as *Bacteroides* and *E. coli* [12,13]. This dysbiosis is a hallmark of CD, and persists across various disease stages and is only partially corrected by a gluten-free diet (GFD) [14,15]. The increase of pathogenic bacteria could play a crucial role in fostering pro-inflammatory responses against gluten, for instance studies have demonstrated that *E. coli* strains obtained from patients with CD exhibit increased virulence characteristics. Furthermore, *E. coli* is implicated in the activation and maturation of dendritic cells (DCs), leading to the enhanced production of pro-inflammatory cytokines, including IL-12 and TNF-alpha, particularly after exposure to gliadin. Conversely, beneficial microbes that are decreased, like *Bifidobacteria*, hold the potential to counteract the adverse effects induced by gluten. *Bifidobacteria* have been shown to mitigate the increased intestinal permeability and the disruption of tight junction (TJ) proteins expression triggered by gluten. Additionally, species of *Bifidobacterium* may play a crucial role in decreasing the production of toxic, immunogenic gliadin peptides within the intestinal lumen, thereby reducing their potential for triggering harmful immune responses [16,17]. The GFD initiates a partial rebalancing of the gut microbiota, yet certain unbalances, especially in microbial diversity, may persist [18].

NCGS patients show a different microbial profile, with a notable decrease in microbial richness indicating a less diverse gut microbiota [19]. This condition is characterized by specific alterations at the phylum level, including the increase of *Ruminococcaceae* and decrease of *Bacteroidetes* and *Fusobacteria*, depicting a picture of a distinct microbial community [20,21]. These microbial differences hint a unique pathophysiological framework for NCGS, distinct from CD, despite the apparent clinical similarity of gluten intolerance. However, the research on microbiota changes in NCGS is still moving the first steps, with findings from limited studies suggesting a need of deeper investigation on how these microbial patterns may influence or reflect the underlying mechanisms of NCGS.

Individuals with food allergies, including WA, exhibit their unique microbial changes, with significant reductions in α-diversity and alterations in β-diversity, leading to a less varied bacterial community [22]. The proliferation of certain bacterial groups such as *Clostridium* and *Anaerobacter*, in contrast with the decline of *Bacteroides* and *Clostridium XVIII*, marks a distinctive microbial signature associated with allergic responses to food [23,24]. The notable decrease in short-chain fatty acids (SCFA), crucial for maintaining gut health, points to a significant disruption in the microbial environment, which may influence or exacerbate allergic reactions [25].

3. Role of dysbiosis in the development and progression of gluten related disorders

CD, NCGS and WA, while distinct in their clinical presentation and immune response, share a common pathway in their relationship with gut microbiota dysbiosis, intestinal barrier function, and immune system modulation.

CD pathogenesis is deeply intertwined with dysbiosis, leading to a cascade of immune reactions against dietary gluten [26]. An unbalanced gut microbiota can contribute to loss of oral tolerance to gluten, partly by increasing intestinal permeability (leaky gut) and facilitating the translocation of gluten peptides that trigger an autoimmune response [27]. The role of gut microbiota in CD extends to influencing the balance of regulatory and effector T cells, exacerbating the inflammatory process. Notably, specific bacteria strains associated with CD may show virulence factors that directly contribute to disease mechanisms, such as increasing intestinal permeability or stimulating adverse immune responses [17]. Additionally, the interaction between dysbiosis and genetic predispositions (HLA-DQ2/DQ8 alleles) further complicates the disease pathophysiology, highlighting a nuanced interplay between genetics, gut microbiota, and immune responses [28].

In NCGS there is an intestinal barrier dysfunction and systemic immune activation in response to the ingestion of gluten and other wheat components, independent from the autoimmune processes seen in CD [29]. Elevated levels of biomarkers of intestinal injury and immune response to microbial products suggest a compromised intestinal barrier that facilitates systemic inflammation [30]. This condition is characterized by a dysregulated gut microbiota that not only contributes to the barrier dysfunction but also interacts with the innate and adaptive immune systems in a different manner from CD [31].

In food allergies the gut microbiota's composition and diversity—or the lack thereof—play crucial roles in modulating the immune system's reaction to food antigens [24]. Dysbiosis in food allergies is associated with reduced microbial diversity, impacting the development and function of regulatory T cells (Tregs) and compromising the intestinal barrier integrity [32]. This compromised barrier facilitates the entry of allergens, triggering allergic responses. Moreover, alterations in the production of immunomodulatory metabolites, such as SCFAs, by the gut microbiota affect the immune response and gut barrier function [33]. The increased prevalence of certain bacteria and a concurrent

decrease in beneficial microbes further illustrate the microbiome's influence on the pathogenesis of food allergies, highlighting the potential role of microbiota-targeted interventions.

In summary, while dysbiosis and intestinal barrier dysfunction are common threads, the specifics of the immune response and the role of the microbiota diverge significantly among gluten-related disorders. CD involves an autoimmune response facilitated by a breach in oral tolerance to gluten, and influenced by genetic predisposition. In contrast, NCGS features systemic immune activation largely driven by innate immunity and barrier dysfunction. The critical role of the microbiota in immune system education and maintenance of tolerance to dietary antigens is highlighted in food allergies, with dysbiosis leading to aberrant immune responses to specific foods[34–37].

4. Mechanisms linking microbiota to gluten related disorders

4.1. Influence of microbiota on gut barrier function

The gut microbiota plays a role in the regulation of gut permeability, immunity and also in the development of appropriate responses to microbial and dietary antigens [38,39]. Various structures and mechanisms prevent microbes in the intestinal lumen from passing into the host's tissues, particularly the mucous layer (through the production of antimicrobial peptides), the intestinal epithelial barrier and the tight junctions.

Mucus, the first barrier between the microbiota and the intestinal tissue, inhibits the passage of pathogenic microbes, however pathogens have developed different strategies to circumvent the mucosal barrier, i.e. by enzymatic degradation of mucus and loosening the tight junctions between cells [40]. On the other hand, the production of antimicrobial proteins by epithelial cells is a mechanism that prevents bacterial penetration through the mucous layer. One of the antimicrobial components is RegIIIγ, a mucosal C-type lectin that interacts with peptidoglycan and exhibits an antibacterial effect for Gram + bacteria [41,42]. Molecules released by inflammatory cells act on the intestinal epithelial barrier (IEB). The breakdown of the IEB allows the entry of antigens and bacterial components into the mucosal layer, thereby initiating the process of inflammation. This results in damage to epithelial cells and dysfunction of tight junctions, along with structural and functional changes in mucus, ultimately resulting in increased intestinal permeability [43,44]. Even though there are several studies on the IEB, to date the sequence linking GRD, as CD, to IEB impairment is still unclear. Data suggest that one of the factors triggering the development of CD is the presence of intestinal barrier alterations, which allow gluten to easily cross the IEB. This alteration allows the transition of gluten into the mucous coat, subsequently activating innate immunity [45]. Zonulin dysregulation is one of the possible mechanisms involved in IEB alterations. Zonulin, a regulator of the intercellular tight junctions, plays a role in the transport of macromolecules implicated in the immune responses and tolerance. The dysregulation of the zonulin pathway can increase the permeability of tight junctions, leading to increased susceptibility to inflammation and autoimmune diseases, as CD [46]. Due to a transcriptional mechanism, zonulin levels are reduced in patients with CD. In addition, it has been shown that the zonulin levels of these patients can revert to normal levels under a GFD [47]. According to some studies, gliadin can bind to receptors for the pro-inflammatory chemokine 3 (CXCR3). This binding triggers the production of zonulin, disrupts tight junctions, reduce the connection of epithelial cells and increase intestinal permeability. Furthermore, there is an over-expression of CXCL10, a ligand for CXCR3, in the small intestine of CD patients [44,48,49]. High levels of zonulin have also been found in the serum of patients with NCGS. A study suggested to measure the serum level of this protein as a biomarker for the diagnosis of this disease and to differentiate it from IBS-D. In addition, zonulin levels were correlated with some typical symptoms of patients with NCGS, such as abdominal distension and pain [50]. In another study it was shown that the administration of *Bifidobacterium lactis* can reduce the increased intestinal permeability induced by gliadin, a component of gluten. This suggests a potential role of probiotics in the treatment of GRD [51].

Gut bacteria play also a role in immune tolerance of gluten through the production of short chain fatty acids (SCFA). SCFA are mostly generated through the metabolism of fibers. They contribute to epithelial growth and regeneration, production of T-reg cells, as well as the pathogenic response of bacteria. They also contribute to maintaining the epithelial barrier through the tight junctions [44,52–54]. In a comparative study, Baldi et al. showed that patients with CD have higher circulating levels of some SCFAs, especially butyric acid, hexanoic acid and decanoic acids, compared to healthy controls, most likely due to an altered intestinal permeability [53]. Several studies have also analyzed the mechanisms involving SCFAs in patients with wheat allergy. In a recent study, it was shown that the administration of *Lactobacillus paracasei AH2* improved some manifestations of WA. This bacterium stimulates the growth of some microbial species involved in the production of SCFAs, such as *Bacteroides and Faecalibaculum.* These changes in the balance of the intestinal microbiota lead to increased levels of SCFA. Furthermore, SCFA are involved in reducing intestinal permeability through the modulation of the tight junctions. In support of this mechanism, high levels of zonulin have been detected after the administration of *L. paracasei AH2*. In this way, *L. paracasei AH2* manages to hinder the passage of allergens, thus reducing the effects of wheat allergy [55]. In another study, the differences in the composition of the intestinal microbiota between patients with food allergies, such as WA, and non-allergic individuals, were investigated. The levels of *Prevotella copri* were much lower in allergic patients as well as the levels of SCFA. However, the diversity highlighted between the two groups could be due to many other factors that were not evaluated in this study [56].

4.2. Microbiota-mediated immune response to gluten

Pathogenic bacteria play a role in the activation of the immune system. The binding between TLR-4 and CD14 specifically identify bacterial endotoxins and lipopolysaccharides present in the wall of bacteria. In this way, they induce the innate immune system to produce different inflammatory cytokines. Indeed, CD14 levels can be used as a marker for the startup of the innate immune system and high levels of this protein are present in patients with CD who do not strictly follow a GFD. This suggests that CD14 can be connected to the pathogenesis of CD, contributing to the mechanisms responsible for the loss of intestinal eubiosis [57–59]. This mechanism of activation of the immune system is also stimulated by the weakening of the intestinal barrier, through which the intestinal immune cells are exposed to alimentary antigens, such as gluten. On the other hand, the activation of inflammatory pathways is prevented by a healthy microbial ecosystem that includes an equal distribution of beneficial and pathogenic bacteria. Beneficial microbes also enhance the tolerance of the immune cells [57,60]. Patients with NCGS express significantly increased serum levels of soluble CD14, the bacterial lipopolysaccharide-binding protein (LPS) and the flagellin. These patients also have high levels of fatty acid-binding protein 2, which is correlated to the alterations in the permeability of the intestinal epithelial barrier. This mechanism could facilitate bacteria translocation, thus activating the immunity response. In addition, it was demonstrated that the levels of these molecules are reduced after a wheat-free diet [61].

Th1 cells specific for gluten antigens release inflammatory molecules, including interferon (IFN-γ), interleukin IL-12, and tumor necrosis factor [62]. Intraepithelial lymphocytes (IELs) undergo cytotoxic transformation when they interact through NK-cell receptors with intestinal epithelial cells. Interleukin-15 (IL-15) is among the inflammatory cytokines that play a primary role in causing the cytotoxic transformation of IELs. Environmental factors, such as persistent exposure to gluten, increase the production of IL-15 and may result in tissue destruction and intestinal atrophy [62–64]. A comparative study

showed that patients with NCGS present an increase of IFN-γ levels after gluten administration for three days. Furthermore, patients with NCGS have high levels of IEL expression even before gluten administration, while the levels of IFN-γ are elevated even before the intake of gluten in patients with CD [65].

Several studies investigated the mechanisms linking the intestinal microbiota to the activation of the immune system and the production of some cytokines in GRD. In a recent, prospective study on young children subsequently developing CD high levels of three inflammatory cytokines, i.e. IFNA2, IL-1a and IL-17E/(IL25), were found. Furthermore, IL-27 and IL-12 levels were increased over time in these patients, suggesting a potential contribution to the systemic inflammation required for the development of CD [66]. Among these cytokines, IL-25 (IL-17E) plays a fundamental role in the activation of the immune system through the regulation of Th-17 cells. The increased levels of this cytokine in the gut could also be caused by the intestinal microbiota, as its levels have been shown to be decreased in antibiotic-treated or germ-free mice [67]. Another study showed that the duodenal microbiota of individuals with active CD exhibits an increased proteolytic activity towards gluten, a finding related to the high numbers of *Proteobacteria*, particularly *P. Aeruginosa*. These findings suggest that protease associated with bacteria, particularly *P. Aeruginosa,* influence immune system through the increased number of immunogenic peptides. In genetically CD-predisposed mice, *P. Aeruginosa* elastase reacts with gluten causing a strong inflammation associated with villus blunting. Elastase generated from *P. Aeruginosa* (LasB) causes an inflammatory effect that is modulated by PAR-2 pathways in mice. This effect does not cause damage on the surface of the intestinal villus and is correlated to the development of intestinal malabsorption since the gluten peptides can activate T-cells [68,69].

The correlation between the immune system and gut microbiota has also been studied in patients with WA. In a mouse model, several features of WA developed after gluten administration, such as intestinal barrier dysfunction, reduced villous height and an increased number of mast cells. The administration of *Pediococcus acidilactici XZ31* reduced the percentage of gluten specific-IgE by shifting the immune system toward the Th1-direction. Furthermore, *Pediococcus acidilactici XZ31* modified the balance of the intestinal flora, increasing the levels of *Bacteroidetes* and reducing the levels of *Firmicutes*. In this way it contributed to reduce the intestinal inflammation, promoting the growth of beneficial bacterial species [70].

In addition to gluten, other wheat components are also responsible for the typical symptoms of patients with NCGS or CD., particularly the amylase-trypsin inhibitors (ATIs). ATIs represent 2–4% of the total proteins of wheat. These proteins stimulate the innate immune response by the activation of the TLR-4 receptors on intestinal macrophages and dendritic cells [71]. In a recent study, Caminero et al. have shown that ATIs induce alteration in the permeability of IEB and also play a role in activating the immune system. Some species of *Lactobacillus* can metabolize ATIs, improving the symptoms of patients with NCGS. However, further studies are needed to understand the pathogenetic mechanisms involved [72].

4.3. Impact of microbial metabolites on gluten digestion and tolerance

SCFAs, particularly butyric acid, have a significant effect on many intestinal functions, as previously described. Intestinal epithelial cells express receptors which are recognized and activated by SCFAs, particularly G-protein coupled receptors (GPR). The production of acid butyric is induced by intestinal microbiota and a specific receptor (GPR109A) is recognized by this metabolite [73,74]. The activation of GPR109A by butyrate and niacin plays a primary role in protecting intestinal cells from inflammation, consequently allowing macrophages and dendritic cells (DCs) to produce anti-inflammatory cytokines, in particular IL-10. Dendritic cells are stimulated through the synergistic effect of histone deacetylases (HDAC) inhibition and GPR109A receptor-induced signaling. This mechanism activates the expression of retinaldehyde dehydrogenase-1 (RALDH1), which is an enzyme involved in the production of retinoic acid. In this way, this metabolite induces macrophages and DCs to stimulate the differentiation of T-reg cells in Th1 direction, which are responsible for IL-10 production [75,76]. In food allergies, such as WA, an altered balance occurs between Th1 and Th2 cells, with a marked increase of cellular differentiation in the Th2 direction. High levels of butyrate in breast milk can suppress the activation of the allergic immune response. Among the mechanisms responsible for this process there is the inhibition of Th2 differentiation by cytokines such as IL-10 and IFN-γ. Furthermore, butyrate acts by reducing intestinal permeability at the tight junctions level and increasing the thickness of intestinal mucus. In this way, this metabolite can reduce the passage of allergens through the IEB, thus inhibiting the activation of Th2 cells [77].

The activity of Foxp3 T regulatory cells is a very important mechanism implicated in the induction of mucosal immune tolerance. The development of Foxp3 regulatory cells in the gut can be stimulated through the polysaccharide-A capsule (PSA) produced by *Bacteroides fragilis.* In this way this bacterium manages to regulate the immune response through the development of a correct balance between Th2 and Th1 cells. Through this mechanism Foxp3-T cells produce anti-inflammatory cytokines in the gastrointestinal tract, e.g. IL-10. Immune responses can be regulated through the regulation of FoxP3+Treg cells. This pathway could be modified to reduce the inflammatory responses occurring in many diseases, such as autoimmune diseases and food allergies [78,79]. In a randomized controlled trial, Konieczna et al. demonstrated that the administration of *Bifidobacterium infantis* in healthy patients induces an increased numbers of FoxP3-Treg cells, resulting in elevated IL-10 production. Furthermore, it was highlighted that tryptophan metabolism, but not retinoic acid metabolism, is required for the induction of Foxp3+T cells after administration of *B. infantis* [37]. The mechanisms implicated in the pathogenesis of food allergies, such as WA, were also shown in a study by Abdel-Gadir et al. In non-allergic individuals the intestinal microbiota is responsible for the induction of ROR-γt + iTreg cells through a Myd88-dependent pathway. This process is implicated in the pathogenic mechanism of food allergies. Due to the loss of balance in the gut microbiota, in allergic individuals there was a reduction in the activation of ROR-γt + iTreg cells, which led to the expression of elevated levels of IgE, that are characteristic of this disease [80]. Moreover, the results of a comparative study conducted by Sapone et al. demonstrated that, unlike patients with CD, patients with NCGS show a reduced expression of FoxP3-A. These results underline that the pathogenesis of NCGS is related to the activation of innate immunity, rather than the adaptive immunity. This suggest that there is an important link between gluten, microbiota and intestinal immune responses, but the involved mechanisms still need to be explored [81].

Several studies have analyzed the mechanisms that correlate the metabolites produced by *Clostridium* species to gluten-related disorders. Among these, the taurodeoxycholate (TDCA) is an inflammatory bile acid which is produced by 7-α-dehydroxylation of taurocholic acid (TCA) from gut microbiota. Specific bacteria involved in this mechanism are *Clostridium XI and Clostridium XIVa* [82,83]. In a recent study it was shown that the most significantly altered metabolites in 5-years old children subsequently developing CD, include 2-Methyl-3-ketovaleric acid, TDCA (taurodeoxycholate), glucono-d-lactone, and isobutyryl-l-carnitine [66]. It was also demonstrated that one of the most involved pathways associated with the functionality of these metabolites is the pentose phosphate pathway. This is responsible for regulating inflammation through the production of NADPH. This antioxidant is found in many mechanisms of regulation, and acts by modulating the reactive species of oxygen [66,84]. Furthermore, in this study TDCA caused villous atrophy in the mouse model and increased the number of $CD4^+$ T cells among IELs. The results of this study suggest that the abundance of TDCA in the intestine plays a role in the development of inflammation, which is involved in the pathogenic

mechanism of CD [66]. In another study Caminero et al. analyzed the activity of the intestinal microbiota in patients with CD and in healthy patients. The findings revealed differences between the two groups, specifically identified in fecal glutenasic activity (FGA) and fecal tryptic activity (FTA). In patients with CD the levels of these two markers were high, unlike healthy individuals. The study focused on the 33-mer peptide that celiacs react to, showing that the activity of this peptide was directly linked to the FGA. Furthermore, *Lactobacillus* species were reduced in patients with CD, unlike healthy individuals, while the *Clostridium* species were increased only in patients with CD. This suggests that an altered balance in the gut microbiota in CD may be responsible for a high proteolytic activity of the gut [85].

The involvement of *Bifidobacterium* species in the underlying microbial origins of CD pathogenesis has gained significant attention in scientific research. In a study conducted by McCarville et al. it was demonstrated that a specific serine protease inhibitor (Srp) produced by the probiotic *Bifidobacterium Longum NCC2705* is able to reduce gliadin-induced inflammation in mouse models of NCGS. Through the comparison between different strains, it emerged that the administration of *B. Longum NCC2705,* which expresses high levels of Srp, could have an important role in the anti-inflammatory activity. The mechanism of serpin (Srp) activity is the inhibition of neutrophil elastase. In this way, the serpins play a role in the reduction of intestinal permeability through the regulation of tight junctions and regulating immune system. These pathogenetic mechanisms are in turn involved in the pathogenesis of GRD and could be modified for complementary treatment of these disease [34].

5. Modulation of microbiota as a therapeutic approach in GRD

To date, the only available treatment for CD is a strict GFD [86]. However, due to its cost and social inconvenience, GFD has a sub-optimal compliance rate [87]. Accidental contamination is common even in the most meticulous patients and can cause intestinal inflammation and persistent villous atrophy [88]. Mucosal healing during a GFD is slow and partial, and nearly 20 % of patients have a non-responsive CD [89]. Given that patients with mucosal recovery have a clinically relevant lower risk of all-cause mortality, this low rate of healing is a matter of concern [90]. To improve mucosal healing and persistent gastrointestinal symptoms, new therapeutic approaches are under scrutiny. The increasing knowledge about the relationship between CD and gut microbiota has led to consider the modulation of the latter as a therapeutic strategy to supplement the GFD.

Probiotics are live microorganisms which, when administered in adequate amounts, confer health benefits to the host [91]. *Lactobacillus* and *Bifidobacterium* are the most represented *genera* in most preparations. Several studies have shown the implication of these *genera* in the development of CD, leading to the consideration of probiotic-based treatments. Despite promising studies on probiotics in animals, human trials are still in their early stage. *Bifidobacterium* spp have been proved to play a role in the regulation of inflammation in CD patients, mostly downgrading the production of pro-inflammatory cytokines. In a double-blinded, placebo-controlled trial, administration of *Bifidobacterium breve (BR03 and B632)* showed a decrease in TNFα production in children with CD on a GFD [92]. In another study, *B. breve* administration led to a gut microbiota modification, by increasing *Actinobacteria* and re-establishing the physiological *Firmicutes/Bacteroidetes* ratio [93]. Moreover, in an animal model of gliadin-induced enteropathy, *Bifidobacterium longum (CECT 7347)* reduced pro-inflammatory cytokines production, such as TNFα, and increased regulatory cytokine production, including IL-10. It also reduced activation of $CD4^+$ T cells, modulating immune responses to gliadin [94]. In a placebo-controlled trial, the administration of *B. longum* in children with CD was associated with the reduction of peripheral $CD3^+$ T lymphocytes and an increased height percentile, reduced number of *Bacteroides fragilis* and sIgA content in stools [95]. In vitro studies on Caco-2 cell line proved that *Bifidobacterium* and *Lactobacillus* can also produce endopeptidases, which digest the epitopes of gluten and reduce inflammatory response to gliadin exposure [96,97]. Furthermore, in patients who consumed hydrolyzed wheat flour (pre-treated with selected *Lactobacilli* and fungal proteases), INF-γ secretion was deemed non-significant when compared to the control group [98]. Hydrolyzation of gliadin can also be obtained by the administration of *VSL#3* mixture [99]. In a mouse model of gliadin-induced enteropathy, *Lactobacillus rhamnosus GG* and *Lactobacillus casei* have shown to play a role in the protection and restoration of mucosal structure [100,101]. Gluten-related enteropathy in mice was also improved by administration of *Saccharomyces boulardii KK1*, which led to a reduction in epithelial cell CD71 expression and local cytokine production [102]. In addition to their immunomodulating effects, probiotics may also alleviate gastrointestinal symptoms, especially in highly symptomatic CD patients on a GFD [103,104].

The literature on other GRD is less extensive. As for NCGS, a pilot study on patients on a GFD showed a reduction in intestinal and extra-intestinal symptoms when *Bifidobacterium longum ES1* was administered [105]. Fermentation of wheat dough (with a mix of *B. longum, L. acidophilus*, and *L. plantarum*) led to gliadin degradation and reduced levels of pro-inflammatory cytokines as a result [106]. Gluten fermentation could be effective in WA as well, where *Lc. lactis LLGKC18* decreased gliadins, glutenins and ATI antigenicity [107]. Moreover, in mouse models of WA, probiotics such as *Pediococcus acidilactici XZ31* and *L. paracasei AH2*, improved intestinal allergic responses, mainly modulating the Th1/Th2 immune balance and gut microbiota composition [108,109].

Additional high-quality trials are still needed to identify and standardize possible future treatments, especially considering the high rate of probiotics use in CD patients [110,111].

6. Prebiotics as a mean to selectively promote beneficial microbial species

Another way to improve the GFD through microbiota modifications is the administration of prebiotics, i.e. substrates selectively utilized by host microorganisms that confer a health benefit [112].

Only few human studies, mostly regarding inulin, have evaluated the role of prebiotics in CD patients. Oligofructose-enriched inulin administration in children with CD on a GFD has shown an increase in *Bifidobacterium* count and SCFA levels compared to placebo [113]. In another study, the same prebiotic led to a decrease in serum hepcidin concentration; this may improve intestinal iron absorption, and the iron deficient anemia that is common in these patients [114]. Oligofructose-enriched inulin administration also showed an improvement in vitamin D and vitamin E status when administered in children and adolescents with CD [115]. Rats fed with an inulin-supplemented GFD showed an increase in calcium absorption, particularly when put on a low-calcium diet [116]. This could improve the often compromised bone state in CD patients. For these reasons, adding prebiotic inulin-type fructans to gluten-free bread seems to be a promising therapeutic approach, providing nutritional and functional benefits to patients [117].

By contrast, a different approach may be required in NCGS patients, where symptoms can be improved by incorporating a low-FODMAP diet with a GFD [118]. Finally, there is no specific literature on WA and prebiotics yet, but given their contribution in allergy prevention and treatment, a future role of prebiotics in WA management cannot be excluded [119].

Further studies are required to better assess the underlying mechanisms of these results and identify the most effective therapeutic approaches.

7. Fecal microbiota transplantation as a novel treatment option

Fecal microbiota transplantation (FMT) is the medical procedure of

transferring human fecal matter from a healthy donor to a recipient. It can be used to treat diseases related to microbiome imbalance and is a well-known effective therapy for recurrent *Clostridioides difficile* infection. It has recently shown promising results in the treatment of various microbiome-related conditions, such as inflammatory bowel disease [120]. To date, there are only a few data of FMT effects in CD. A case-report described a patient with refractory CD type II and recurrent *Clostridioides difficile* infection, showing a resolution of the infection and an improvement of the CD symptoms after FMT. Notably, at the six-months follow-up, duodenal biopsies indicated a complete recovery of the villous atrophy [121]. Additionally, there is an ongoing clinical trial by the Chinese University of Hong Kong aimed at exploring the safety and efficiency of FMT in several autoimmune and inflammatory disorders, including CD. The trial is still recruiting, and no data have been released yet [122].

To date no studies have been conducted on FMT and other GRD. Nevertheless, given the overlap between NCGS and IBS symptoms and the current studies on FMT efficacy in IBS patients, it is possible that FMT will play a role in managing NCGS as well [123,124].

8. Conclusions

Fig. 1 shows the differences in intestinal microbiota composition and pathophysiological mechanisms involved in the different GRDs. In untreated CD the microbiota is characterized by a reduction in beneficial microbes like *Lactobacillus* and *Bifidobacterium* and an increase in pathogenic ones such as *Bacteroides* and *E. coli*. Dysbiosis is a hallmark of CD, persists across various disease stages and is only partially corrected by a gluten-free diet. NCGS patients show a different microbial profile, with a notable decrease in microbial richness, and an increase of *Ruminococcaceae* and decrease of *Bacteroidetes* and *Fusobacteria*. The increase of certain bacterial groups such as *Clostridium* and *Anaerobacter*,

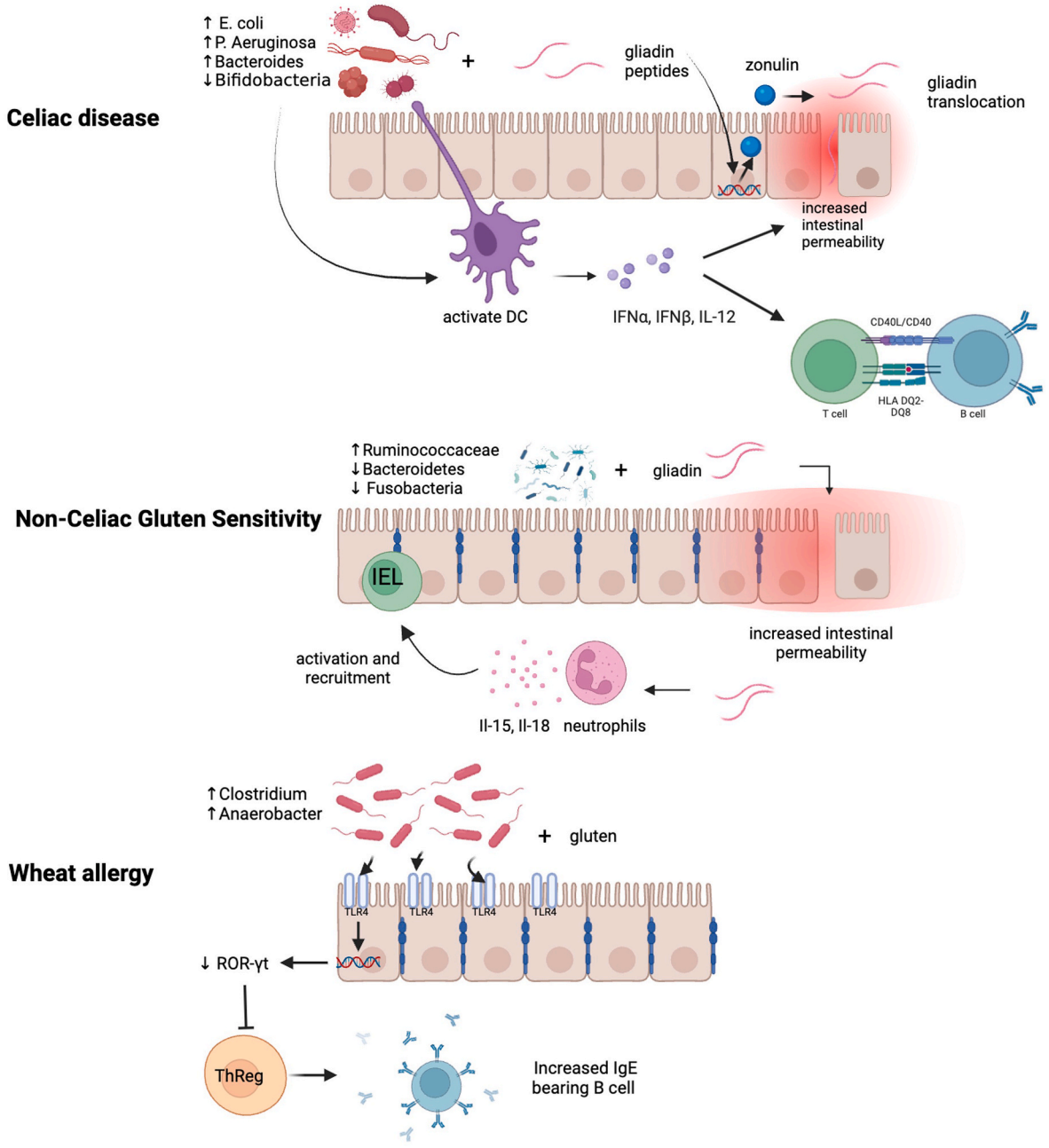

Fig. 1. Different microbiota composition and pathophysiological mechanisms involved in gluten-related disorders.

in contrast with the decline of *Bacteroides* and *Clostridium XVIII*, marks a distinctive microbial signature associated with allergic responses to food. Mechanisms linking the gut microbiota to the development of GRD include effects on the gut barrier function, microbiota-mediated immune response to gluten, and an impact of microbial metabolites on gluten digestion and tolerance.

Although the gluten-free diet is the primary therapy of GRDs, treatment with probiotics may contribute to improve the natural history of these disorders, for instance by minimizing the damaging effects of gluten contamination and accelerating the catch-up growth at the beginning of the dietary treatment of CD. Additional high-quality trials are still needed to identify and standardize the use of probiotics/prebiotics in GRDs.

Practice points

- Gut microbiota composition and function may be involved in the pathogenesis of GRDs
- Dysbiosis is a hallmark of CD, with a reduction in beneficial microbes like *Lactobacillus* and *Bifidobacterium* and an increase in pathogenic ones such as *Bacteroides* and *E. coli.* It persists across various disease stages and is only partially corrected by a gluten-free diet.
- NCGS patients show a different microbial profile, with a notable decrease in microbial richness, and an increase of *Ruminococcaceae* and decrease of *Bacteroidetes* and *Fusobacteria.*
- The increase of certain bacterial groups such as *Clostridium* and *Anaerobacter*, in contrast with the decline of *Bacteroides* and *Clostridium XVIII*, marks a distinctive microbial signature associated with allergic responses to food such as WA.
- Mechanisms linking the gut microbiota to the development of GRD include effects on the gut barrier function, microbiota-mediated immune response to gluten, and an impact of microbial metabolites on gluten digestion and tolerance.
- Although the gluten-free diet is the primary therapy of GRDs, treatment with probiotics may contribute to improve the natural history of these disorders, for instance by minimizing the damaging effects of gluten contamination and accelerating the catch-up growth at the beginning of the dietary treatment of CD.

Research agenda

- Future studies are needed to clarify whether specific alterations of the intestinal microbiota may trigger the development of gluten-related disorders, particularly celiac disease;
- It will be important to investigate the possibility that an early intervention aimed to correct the dysbiosis found in gluten-related disorders may change the natural history of these conditions;
- Further intervention studies should investigate the role of probiotics/prebiotics in the treatment of gluten-related disorders, particularly during the initial phase of treatment of celiac disease and to minimize the effects of possible transgression to the primary treatment, i.e. the gluten-free diet.

CRediT authorship contribution statement

Giulia Catassi: Writing – original draft, Supervision, Methodology, Data curation, Conceptualization. **Elena Lener:** Writing – original draft, Data curation. **Maria Maddalena Grattagliano:** Writing – original draft, Data curation. **Sofya Motuz:** Writing – original draft, Data curation. **Maria Antonietta Zavarella:** Writing – original draft, Data curation. **Stefano Bibbò:** Writing – original draft, Data curation. **Giovanni Cammarota:** Writing – review & editing, Methodology, Conceptualization. **Antonio Gasbarrini:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Gianluca Ianiro:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Carlo Catassi:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

C.C reports personal fees for consultancy for Dr. Schar Food; A.G. reports personal fees for consultancy for Eisai S.r.l., 3PSolutions, Real Time Meeting, Fondazione Istituto Danone, Sinergie S.r.l. Board MRGE and Sanofi S. p.A; personal fees for acting as a speaker for Takeda S. p.A, AbbVie and Sandoz S. p.A; and personal fees for acting on advisory boards for VSL3 and Eisai. G.I. has received personal fees for acting as the speaker for Alfa Sigma, Biocodex, Illumina, Malesci, Sofar and Tillotts Pharma and for acting as consultant/adviser for Biocodex, Malesci, and Tillots Pharma. The other authors have no potential competing interest to disclose.

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