Chapter 24

Intestinal Microbiota and Celiac Disease

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Abstract

Intestinal microbiota is considered to perform important metabolic and immunologic functions, which affect the host's health and disease risk. Evidence from epidemiologic studies suggests that environmental factors influencing the intestinal ecosystem, such breast-feeding practices and incidence of gastrointestinal infections, can also contribute to the risk of developing celiac disease (CD). Breast-feeding seems to exert a protective role against CD and it also favors bifidobacteria colonization in the infant's gut. Colonization of the newborn intestine is considered a critical stimulus for the adequate development of immune and intestinal barrier functions, modulating host protection mechanisms against allergens and pathogens. Observational studies indicate that gut colonization patterns of infants at genetic risk of developing CD differ from those of non-risk infants, which could also influence CD development. Imbalances in the gut microbiota of CD patients in comparison to healthy controls have also been reported in several observational studies. It is hypothesized that these alterations and specific bacteria isolated from patients could contribute to CD pathogenesis by activation of the pro-inflammatory Th_1-type response typical of the disease according to *in vitro* and animal studies. Therefore, dietary interventions based on the use of probiotics are being considered as potential adjuvants and preventive strategies to control the disease, as well as to improve quality of life of CD patients. These strategies could theoretically contribute to restoring the intestinal ecosystem, thereby ameliorating the severity of CD pathological manifestations and to developing a gluten-tolerant phenotype in subjects at risk via different mechanisms.

1. Introduction

Celiac disease (CD) is a chronic autoimmune enteropathy, caused by an intolerance to gluten proteins in cereals, including wheat, barley, rye and possibly oats, that causes severe functional and morphological alterations of the small intestinal mucosa. Typical cases of this disease usually occur in the first years of life, frequently manifested with gastrointestinal symptoms; however, extra-intestinal or atypical manifestations are increasingly more frequent, especially later in life. CD is also associated with other immune-based diseases such as dermatitis herpetiformis, IgA deficiency, diabetes mellitus type I, thyroiditis and autoimmune hepatitis.[1,2]

Genetic and environmental factors (mainly gluten) play a role in this pathology; however, other variables such as breastfeeding practices, incidence of gastrointestinal infections and intestinal microbiota composition could also be involved, as outlined in Figure 1.[3-5] Genetic susceptibility to CD is determined by the specific class II major histocompatibility complex (MHC) HLA-DQ encoding HLA-DQ2 or HLA-DQ8 heterodimers involved in antigen presentation. Most of celiac patients express HLA-DQ2/DQ8 molecules, indicating that it is a necessary factor for the disease development; however, these risk factors are also present in 30% of the general population and only a low percentage develops CD, indicating that their presence is not sufficient for the disease to become manifest. Studies on twins have also shown that in 25% of cases one twin does not develop CD,[6] indicating that other environmental factors besides genotype are also involved in the development of this disease.

In recent years imbalances in the intestinal microbiota composition of CD patients and in individuals at risk have been detected.[3,7,8] The colonization process in the early stages of life and the interaction between intestinal microbiota and the innate and adaptive immune systems in different stages of life could be crucial for the development of oral tolerance to gluten proteins and to determine the risk and severity of this pathology.

Currently, the only treatment for CD is a strict, lifelong gluten-free diet. Although symptoms usually resolve after following this dietary strategy, its maintenance is difficult due to the presence of gluten in most processed foods. In addition, a percentage (3-5 %) of patients have refractory CD and do not respond to this dietary pattern (reviewed by Mooney et al.[9]). This increases the need for developing additional preventive and therapeutic strategies to the gluten-free diet. Among these, we could include the use of proteolytic enzymes that hydrolyze the ingested gluten, intestinal permeability modulators and peptide-based vaccines with specificity for HLA-DQ2 molecules that facilitate desensitization to gluten as well as nutritional intervention based strategies, including food ingredients with immunomodulatory properties and a positive influence on the intestinal barrier function.[10]

2. Intestinal Microbiota, Breastfeeding and HLA-DQ Genotype

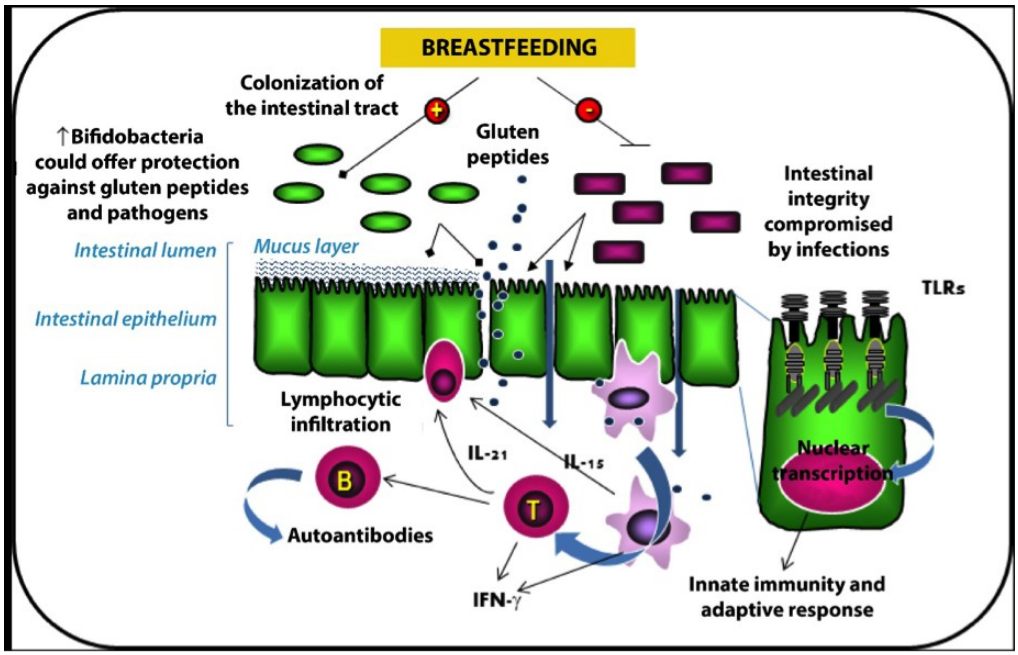

Figure 1. Influence of breastfeeding and intestinal microbiota on celiac disease pathogenesis.

Among the environmental factors related to CD etiology, besides gluten intake, we can include breastfeeding practices, timing of dietary gluten introduction, the incidence of infections and intestinal microbiota composition.[3,11,12] Epidemiological studies indicate that breastfeeding may have a protective effect against the development of CD.[13] Several studies have identified the presence of microorganisms and prebiotic oligosaccharides in breast milk and have described its effect on the composition of the infant gut microbiota and on the immune system modulation, which could also influence the risk for certain diseases (reviewed in Fernández et al.[14]). In breast-fed children, bifidobacteria dominate the intestinal microbiota, while artificial feeding promotes the colonization of a more heterogeneous microbiota which is similar to that of the adult population.[15,16] Furthermore, the comparative analysis of stool samples from twins, adults and children with different kinship degrees has led to the conclusion that genotype also affects the intestinal microbiota composition.[17-20] Toivanen et al.[21] pointed out that certain MCH genes might be involved in differences in fecal microbiota observed in mice with different genetic backgrounds.

In the context of CD, a prospective study of a cohort of new-borns with CD risk due to their family history using real-time PCR showed that both the type of breastfeeding as well as the HLA-DQ genotype influence the intestinal colonization process.[4,22] In children with a high risk disease, irrespective of breastfeeding practices, a reduction was observed in the number of *Bifidobacterium* spp. and in the species *B. longum*; however, breastfeeding attenuated the

differences and favored colonization by species of this genus. An increase in the number of *Staphylococcus* spp. associated with increased genetic risk in infants fed with maternal and artificial formula was also observed. Furthermore, an increase was detected in the number of *B. fragilis* groups associated with genetic risk but only in children fed with formula.[4] In a subset of this cohort, colonization by species of the genus *Bacteroides* was also assessed using DGGE and showed that species diversity was higher in artificially fed infants than in breastfed infants.[22] Prevalence analysis, considering only the feeding type, showed that the intestinal microbiota of formula-fed infants was characterized by the presence of *B. intestinalis* and those who had been breast-fed by the presence of *B. uniformis*. Furthermore, analysis as a function of the genotype showed greater species diversity in low-risk infants than in the high-risk ones and increased prevalence of the *B. vulgatus* and *B. uniformis* in high and low risk infants, respectively. When considering the feeding type and genetic risk variables together, it was concluded that the prevalence of *B. uniformis* characterized the intestinal microbiota of children at low genetic risk and was favored by breastfeeding. Overall, it was observed that breastfeeding attenuated microbiota differences related to genotype, which could partly explain the protective effect that has been attributed to breastfeeding on the development of CD.

3. Infections and Celiac Disease

Some epidemiological studies have linked the incidence of infection of bacterial or viral origin, with the risk of CD. Several hypotheses have been proposed to explain the association between the incidence infections and CD, including the similarity between the bacterial or viral antigens and immunogenic gliadin peptides that could cause a similar reaction, and an over-stimulation of the immune system secondary to an infection with production of inflammatory cytokines such as TNF-α, INF-γ or IL-15 (reviewed in Jabri and Sollid[23]).

A study performed in Switzerland, which analyzed perinatal data from more than three thousand children who had developed CD showed that the main risk factor for its occurrence had been exposure to infections during the neonatal stage.[24] A subsequent study focused on establishing an association through serum level differences in antibodies to some infectious agents between healthy individuals and celiac patients. The results showed a lower IgG antibody prevalence in celiac patients, suggesting that infections by the three tested viruses (rubella, cytomegalovirus and Epstein-Barr virus) could exert a protective effect on the development of CD.[12]

Kagnoff et al.[25] proposed that the emergence of CD could be triggered by a type-12 adenovirus infection due to the similarity alpha-gliadin exhibits with this virus' E1d protein. The detection of an increase in IgG antibodies against the E1d protein in the sera of children with CD compared to the levels in the control group, seemed to support this hypothesis.[26] However, other studies have come to conflicting conclusions. Thus, Howdle et al.[27] found no difference in the serum levels of this protein between celiac patients and controls. Another infectious agent, which has been associated with CD in epidemiological studies, is hepatitis C. This association was based on the fact that the incidence of chronic liver diseases is 15 times higher in CD patients than in the non-celiac population[28], and in 5% of the cases, the onset of autoimmune liver diseases are accompanied by CD.[29] However, even though this virus is considered to be able to trigger

secondary autoimmune processes, studies do not indicate an increase in CD in hepatitis C patients[30] and the association might simply be casual.[31] A prospective study of 1931 children with the CD risk genotype indicated that a higher rotavirus infection incidence (based on detection of positive serum antibodies against this pathogen), increased the risk of the disease. Similarly, studies associated CD with *Campylobacter jejuni*[32] and *Giardia lamblia*[33] infections in individual case studies. These observations seem to suggest the possible involvement of gastrointestinal infections in triggering CD, through increased intestinal permeability or amplification of the immune response to gluten peptides.

4. Intestinal Microbiota in Celiac Disease Patients

In recent years alterations in the intestinal microbiota composition of biopsies and feces from children and adults with CD have been detected compared to those of controls.[7,8,34] Microbiological analysis of duodenal biopsies by *in situ* hybridization techniques and flow cytometry showed that the ratio of Gram-positive to Gram-negative bacteria in CD patients, at the time of diagnosis and after treatment with a gluten-free diet for at least 2 years, was inferior than that detected in control individuals, as well as the ratio of potentially beneficial bacteria (*Bifidobacterium* + *Lactobacillus*) to potentially harmful ones (*E. coli* + *Bacteroides*).[7] Analyses by real-time PCR, have shown that the number of *Bacteroides* spp. in the duodenal and fecal microbiota of CD patients (treated or not with the gluten-free diet) was higher than those detected in control individuals.[34] The number of *E. coli* and *Staphylococcus* spp. was also higher in untreated patients compared to controls, but their concentrations were normalized after following a gluten-free diet. *Bifidobacterium* spp. and *B. longum* concentrations in CD patients' feces and biopsies were lower than in controls, although the differences between biopsies were statistically significant only between patients at the time of diagnosis and controls.[8]

Analysis of phylogenetic groups and gene prevalence associated with virulence factors in isolated enterobacteria from stools of CD patients and healthy children have also shown significant differences.[35] Analysis of phylogenetic groups (A, B, C and D) of *E. coli* clones showed that the control group had no differences in their proportion, while in the two groups of children with CD commensal isolates (A and B1) belonged mainly to phylogenetic group A. The virulent clone distribution represented by phylogenetic groups B2 and D, also exhibited differences between the two groups of children with CD; isolates of the group B2 were more abundant in patients with active CD and isolates of the group D were more abundant in CD patients treated with the gluten-free diet. Other authors also described an increased prevalence of virulent phylogenetic groups, especially the group B, in patients with Crohn's disease and ulcerative colitis.[36] In addition; *E. coli* clones belonging to virulent phylogenetic groups (B2 and D) from children with CD in active and non-active phases carried higher numbers of genes encoding virulence factors than those isolated from the control group. The prevalence of genes encoding for *P fimbriae*, K5 capsule and hemolysin was significantly higher in both CD patient groups than in healthy children. These results suggest that the enteric microbiota of CD patients have a higher pathogenic potential than in healthy subjects, which could favor the disease development or aggravate the disease symptoms.[35] Analysis of *Staphylococcus* isolates in a selective culture medium has also shown that children with CD, treated and untreated with a gluten-free diet,

have greater S*taphylococcus epidermis* abundance with methicillin resistance genes, which is one of the main pathogens involved in nosocomial infections.[37] Finally, analysis of isolates from the genus *Bacteroides* has allowed detecting an increase in the species *B. fragilis*, which produces metalloproteases and is involved in opportunistic infections, in treated and untreated celiac patients compared with healthy subjects.[38]

Overall, these studies indicate that there are imbalances in the composition of the intestinal microbiota of CD patients compared with controls; the fact that these alterations are only partially restored after adherence to a gluten-free diet indicates that they are not only a secondary consequence the inflammatory process associated with active phase of the disease and it could play a greater role in its etiology and pathogenesis.

5. Pathogenic Mechanisms of gut microbiota

Oral tolerance to food components is a biologically complex process resulting from the interaction between environmental and individual genetic factors and that may depend on age, dose and postnatal antigen contact period, antigenic composition and structure, intestinal barrier integrity and degree of mucosal immune activation.[39,40] The mechanisms by which intestinal microbiota alterations could contribute to the etiology and pathogenesis of CD include (i) alterations of the microbiota interaction with epithelial and immunocompetent cells leading to activation of signaling mechanisms and inflammation mediators, (ii) alteration of the microbiota's ability to degrade or reduce the glycocalyx and secreted mucus that will influence the intestinal epithelium's barrier properties and (iii) the possible translocation of potentially pathogenic bacteria or derived molecules to the lamina propria.[41,42]

In situ studies of rat intestinal loops show that the presence of potentially pathogenic bacteria (*E. coli* CBL2) or real pathogens (*Shigella*) aggravates the intestinal permeability alterations caused by gliadins and facilitates their translocation to the lamina propria.[43] Under physiological conditions, the intestinal epithelium is a nearly impermeable barrier to macromolecules; however, CD is associated with an increased intestinal permeability[44], which facilitates access of gliadin-derived peptides to the lamina propria and their interaction with the components responsible for the immune response. Gliadins, as it is the case for some pathogens, cause alterations in intercellular tight-junction-related proteins and re-organization of different molecular components (zonulin, occludin, cadherin and claudins).[45] The re-organization of the tight-junction related proteins and the increased paracellular permeability occurs along with the inflammatory response characterized by the production of cytokines such as tumor necrosis factor α (TNF) and interleukin 1 β (IL-1β). They have an important function in further promoting increased intestinal permeability and lymphocyte infiltration[46,47] and activation of the nuclear factor kappa-B (NfkB) pathway.

The influence of the host genotype and the microbiota on the intestinal epithelium glycocalyx composition has also been considered to be a possible pathogenesis mechanism in the context of CD. The intestinal epithelium glycocalyx has an important role in preventing direct contact of ingested compounds and intestinal pathogens with epithelial cells.[43] Previous studies have

demonstrated alterations in the rate and/or composition of glycoconjugates, which compose the glycocalyx and mucus layer in CD patients.[49] CD patients have a high proportion of D-galactose and α (1,2)-fucose residues, while these residues are not found in the mucosa of healthy individuals[49], who do have β-gal(1→3)galNAc residues.[50] Thus, it has been suggested that particular glycosylation patterns could promote the adhesion and colonization of a specific microbiota and pathogens. However, it has also been postulated that these changes in glycosylation patterns could be induced by alterations in the intestinal microbiota. Several studies have shown changes in the fucosylation and/or galactosylation patterns of different intestinal epithelium glycoconjugates in diverse animal models as a function of gut colonization.[51-53] However, there is a lack of studies concerning the particular role of host genotype and microbiota composition in glycosylation patterns and CD risk.

The mucus layer secreted into the luminal medium constitutes a physical barrier for dietary antigens and for intestinal commensal and pathogenic bacteria. This barrier depends largely on the mucus composition in different mucines.[41,42] E*x vivo* studies have demonstrated higher expression levels (mRNA) of type 2 mucin (MUC2) in CD patient biopsies compared to biopsies from treated CD patients.[50] MUC2 biosynthesis and secretion is a process that has been associated with a possible bodily defense mechanism against infections by intestinal pathogens[54,55], which also limits the proportion of commensals being in contact with the epithelial mucosa.[55] However, the increased MUC2 expression in CD patients has also been associated with globet cell metaplasia[50] related intestinal mucosa atrophy and damage.[56] In rat intestinal loops it has been shown that gliadins reduce the number of mucus-producing cells and that this reduction is even more pronounced in the presence of intestinal pathogens (*Shigella* CBD8) and potential pathogens (*E. coli* CBL2).[43]

It has also been proposed that intestinal dysbiosis detected in individuals with CD may result from an alteration in the host's antimicrobial peptide production, such as defensins (HD5 and HD6).[50] However, another study conducted in adults with CD treated with a gluten-free diet demonstrated a lower HD1 expression in duodenal biopsies, while that of HD2, -3 and -4 did not show significant changes.[57] Defensin production is essential in host defense mechanisms and modulates the intestinal ecosystem composition.[58,59] These peptides are produced in response to bacterial antigens such as Gram-negative bacteria lipopolysaccharide (LPS) and Gram-positive peptidoglycan (muramyl dipeptide).[60] Although in CD patients fewer defensin-encoding gene copies have been detected, this is not always related to a reduction in the final production of active peptides.[61]

Toll like receptors (TLRs) have a crucial role during the development of the innate immune response to environmental antigens as well as in the discrimination between commensal bacteria and intestinal pathogens.[62] The stimulation of different TLRs activate signaling pathways and regulate the expression of various genes and inflammatory cytokines conferring them a critical role in the activation and severity of the innate immune response. The response to these stimuli appears to be associated with the interaction of histocompatibility molecules (MHC-II) contributing to the maturation of T "helper" lymphocytes.[63] Recent studies have suggested the involvement of TLRs in CD.[64-66] In these studies, an increased TLR2[64-66] and TLR9[65] expression is reported, while the effects on TLR4 expression are more controversial.[64-66] In no case significant alterations have been reported in expression of TLR3[65,66] (activated by viral RNA) and/or TLR5[65] (activated by bacterial flagellin). However, recent *in vivo* studies have demonstrated the critical

role of IFN-α/β production in the activation and maturation of T CD4+ and CD8+ cells, in the initial stages of viral infections.[67] An increased TLR2 and TLR4 expression has also been detected on dendritic cells and monocytes of children with CD even after treatment with a gluten-free diet.[68] Several studies seem to suggest that molecular signaling through these receptors, mediated by interactions with bacterial components such as LPS from Gram-negative bacteria, may contribute to the activation and severity of the innate immune response in CD and to the enteropathy. Further, various components of the TLR family associated with the MyD88 molecular signaling pathway, are potent inducers of type I IFN production with subsequent activation of other inflammatory inducible genes in response to microbial and/or viral stimuli.[69] This interaction could contribute to the T cell-mediated immune response.[70] Besides, diverse pro-inflammatory cytokines such as IL-6, TNFα and IFN may promote the development of autoimmune processes.[71]

The possible influence of alterations in the intestinal microbiota composition on the inflammatory process typical of CD has been evaluated through *in vitro* studies.[72] In this study it was found that the fecal microbiota of CD patients induced an increased *in vitro* production of inflammatory cytokines in peripheral blood mononuclear cells (PBMCs) than that of healthy subject, which could contribute to the development of the Th1 type cytokine profile characteristic of CD. Subsequent studies confirmed that enterobacteria (*E. coli* CBL2 and *Shigella* CBD8), isolated from CD patient feces could trigger IL-12 and/or IFN-γ secretion associated with an increase in HLA-DR and CD40 molecule expression in PBMCs.[73] These results suggest that certain components of the altered microbiota of CD patients could contribute, together with gluten peptides, to the inflammatory process of CD. Using an intestinal loop animal model, co-inoculation of *E. coli* CBL2, gliadins and IFN-γ reduced the production of metalloproteinases inhibitor (TIMP-1) and an increased vascular endothelial growth factor secretion (VEGF).[73] In addition, recent *in vitro* studies suggest a potential role for different *Bacteroides fragilis* strains, which exhibit virulence factors that may favor epithelial permeability alteration and contribute to the production of potentially inflammatory peptides from gliadins, in CD.[38]

In general, existing scientific evidence suggests partial convergence of the pathogenic mechanism of action of gluten peptides and of potential pathogenic intestinal bacteria in CD, which could aggravate the inflammatory response and the intestinal permeability alteration.

6. Potential Probiotic Protection Mechanisms

Based on established associations between CD and intestinal dysbiosis, the possibility of using intervention strategies in the intestinal ecosystem, based on administration of probiotics[3,74] has been suggested for health restoration and for reducing the risk of disease in these patients. Probiotics are defined as live microorganisms which, when administered in adequate amounts, exert a beneficial effect on the host.[75] Among the probiotic mechanisms that could contribute to the acquisition of oral tolerance to dietary antigens, to reducing the severity of CD manifestation, and to health recovery in diagnosed patients, we can include the immunomodulatory effects and the ability to hydrolyze and reduce the toxicity of gliadin-derived peptides, to improve intestinal barrier function and restore intestinal microbiota composition.

Comparative studies of germ-free and conventional animals suggest that gut colonization by microbiota is necessary for the proper development of mucosal and systemic immune responses, such as the production of immunoglobulins and antigens.[76] Studies on some probiotic strains indicate that they have an important role in various processes that depend directly on mucosa-associated lymphoid tissue, such as oral tolerance to environmental antigens and to the commensal microbiota and the release of chemokines and cytokines that determines the balance of Th1/Th2 lymphocyte populations.[77] Besides, they can also participate in the innate response through their interaction with TLRs expressed by epithelial cells and antigen-presenting cells. Within the CD context, studies that evaluate the immunomodulatory capacity of probiotic strains or potentially beneficial bacteria are relatively scarce.[74,78-80] The transgenic mouse model expressing HLA-DQ8 molecules, sensitized with gliadin and adyuvant[74,78,79] that develops a characteristic Th1 cellular response although without intestinal mucosa damage, has been used to assess the effect of different *Lactobacillus* species (*L. paracasei, L. fermentum* and *L. casei*) and *Bifidobacterium lacti*s. These studies have shown that strains of these species have an activating rather than a suppressive effect on the innate and adaptive immune responses. It has been shown that these lactobacilli favor maturation of the immature bone marrow dendritic cells isolated from these animals *in vitro* and that some of the strains also favor TNF-α production upon gliadin stimulation in both *ex vivo* and *in vivo* experiments.[79] In addition, *L. casei* administration to sensitized animals potentiated the CD4+ T cell response against gliadins. In this context, it has been suggested that the strain *L. casei* ATCC 9595 could be used as a vaccine adjuvant for promoting cellular immune response.[78] In another study, the administration of the strain *Bifidobacterium longum* CECT 7347 to lactating rats sensitized with IFN-γ intraperitoneally and fed with gliadin,[80] led to partial enteropathy reproduction.[80,81] In this model, bifidobacteria administration resulted in a lower systemic proportion of CD4+ cells and CD4+Foxp3+ (regulatory T cells) and reduced the TNF-α production and increased IL-10 production in the small intestine compared to the disease model fed with placebo. IL-10 production plays a key role in modulating the cellular response triggered by gliadins, reducing IFN-γ production and antigen-specific cellular proliferation and inducing regulatory T cells.[82,83]

In this respect, *in vitro* studies also showed that different bifidobacterial strains (*B. longum* CECT 7347 and *B. bifidum* CECT 7365) have a positive effect favoring IL-10 production and inhibiting IFN-γ in PBMCs.[72] Subsequent *in vivo* studies with an animal intestinal loop model showed that *B. bifidum* CECT 7365 promotes the proliferation of globet mucus-producing cells, whose numbers are reduced by increased IFN-γ secretion in the context of CD.[43] In addition, bifidobacteria and IFN-γ co-administration caused no observable adverse effects regarding the zonulin-1 expression and increased chemotactic factors (MCP-1) and metalloproteinase (TIMP-1) inhibitors production, reducing the tissue damage caused by IFN-γ. On the other hand, *in vitro* studies have shown that the strain *B. longum* CECT 7347 is capable of increasing the gliadin digestion degree leading to the generation of peptide patterns with a lower inflammatory potential during gastrointestinal digestion.[84] Other studies have also shown that different species of the genus Rothia, mainly present in the oral cavity, have proteolytic activity on gliadins, but their possible *in vivo* effect is unknown.[85,86]

The immunomodulating potential of some probiotics has also been demonstrated in other inflammatory and autoimmune pathologies. In mice which reproduce an experimental colitis model it has been demonstrated that some probiotic strains, able to induce *in vitro* an increased IL-10 production and a reduced IL-12 production, exert an *in vivo* protective effect against

colitis.[87] Likewise, the positive effects of the probiotic product VSL#3 on an autoimmune diabetic mouse model have been demostrated.[88] In humans, certain probiotics have also demonstrated their utility in pouchitis remission, although their efficacy is debatable in patients with ulcerative colitis and especially with Crohn's disease.[89]

In the context of CD, studies performed *in vitro* and in experimental animal models suggest that strains such as *B. longum* CECT 7347 could exert protective effects favoring anti-inflammatory and regulatory cytokine synthesis, reducing gliadin-mediated inflammatory and toxic response and microbiota alterations; however, human studies with an adequate experimental design are needed to assess the efficacy that the bacterium evaluated in pre-clinical tests may confer to the patients.

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