Gluten-hydrolyzing probiotics: An emerging therapy for patients with celiac disease (Review)

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Abstract. Celiac disease (CD), also known as gluten-sensitive enteropathy, is an autoimmune disorder characterized by variable malabsorption syndrome with characteristics, such as chronic diarrhea, weight loss and abdominal distention. Possible therapies for CD include dietary and non-dietary strategies; the latter include permeability inhibition and tissue transglutaminase (tTG) blockage using chemotherapeutic drugs. Dietary strategies for the management of CD include a gluten-reduced diet, and the supplementation of probiotics and their products. The gluten-reduced diet is not always sustainable due to the availability of gluten-free nutritional commodities. In this context, probiotics are live microorganisms and their products are supplemented to the patients in order to improve their overall well-being. The effects of probiotics on gut health varies from species to species, and it is dependent on environmental factors and other commensals present in the gut. The ameliorating effects of probiotics include the detoxification of gluten peptides, the strengthening of the intestinal epithelial barrier and the degradation of toxin receptors, adhesion to intestinal mucosa, the competitive exclusion of pathogens, the production of inhibitory substances against pathogens, and the regulation of immunity and attenuation of the inflammation associated with a Toll-like receptor through immunomodulation. These observations suggest that a combination of specific gut microbiota and probiotics may prove to be beneficial for patients with CD. In this context, the present review focuses on the prevalence and implications of the disease, as well as the mechanisms of the effects of probiotics, which may aid in the development of natural food adjuncts for individuals prone to or with CD.

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Contents

1. Introduction

Celiac disease (CD) is an unusual malabsorption syndrome, an autoimmune enteropathy among genetically susceptible individuals. CD has been known as an abdominal disorder and has long been listed in the medical lexicon. It was first described in the first century A.D. by Aretaeus Cappadocia, a contemporary of the Roman physician, Galen, using the Greek term 'koeliakos' (suffering of the bowels) (1). At a later date, Samuel Gee (1880), a British physician, defined CD as a type of chronic indigestion in humans (2). The cause of the disease, specifically distinct from other digestive disorders and their symptoms, has been studied over the past 4 decades (3). Initially, it was reported to be prevalent in western part of the world, although its distribution is found globally. This disease has become a very common lifetime disorder among individuals with a prevalence of 0.5 to 1% worldwide (4). However, the World Gastroenterology Organization (WGO) suggests that the female to male for CD is 2:1. Serological studies using anti-gliadin, anti-endomysium or anti-transglutaminase antibody assays, which are hallmark tests for CD, have demonstrated a high prevalence of CD noted in the Middle East, North Africa and India (5,6). In India, the number of cases with CD has exhibited an increasing trend recently and perhaps coincides with a large intake of gluten-rich foods (6). Currently, due to the prevalence of diabetes, Southern Indians also prefer wheat as a staple food, unlike previously. This may also be one of the reasons for the observed increase in the number of cases of CD in India. Therefore, the Indian Task Force for Celiac Disease directed and encouraged research on the prevalence and diagnosis of CD. It has also made the

regulations for the marking of gluten-free/reduced products and subsidies on these foods, and has stated that these foods should be sold at a reasonable price in order to be widely available to patients with CD (7,8). Moreover, there is a great demand for gluten-free/reduced food for the prevention and management of CD in India. In this regard, the present review article highlights the exploration of gluten-hydrolyzing probiotics as another important management strategy for patients with CD.

2. Implications of celiac disease on human health

CD, is referred to by various terms, such as celiac sprue, non-tropical sprue, idiopathic sprue, idiopathic steatorrhea and gluten enteropathy. CD is observed as a permanent intolerance to the storage proteins of wheat, rye and barley among human leucocyte antigen (HLA)-DQ2/DQ8-positive individuals (9). This condition has been characterized by complex adaptive and innate immune responses that yield characteristic chronic inflammation and villous atrophy in the small intestine, as well as systemic inflammation with the deposition of disease-specific auto-induced antibodies in various parts of the body (10). CD can manifest in individuals with a previously unexpected range of clinical symptoms consisting of malabsorption syndrome with chronic diarrhea, weight loss and abdominal distention, affecting the intestine as a primary site of the disease, leading to the destruction of any organ and/or the digestive system of the body and consequent multisystemic disorder (3,11). In addition, CD has been associated with a number of complications, mainly including malignancy and autoimmune disorders (12), which can lead to an improper diagnosis with tropical sprue, particularly due to overlapping or with atypical symptoms. CD can be diagnosed by the detection of autoantibodies generated upon the ingestion of gluten or by a bowel biopsy examination. In addition to this, the incidence of CD has been found to be associated with diabetes or hypothyroidism, and or chronic liver disease when compared to tropical sprue (7). Typical disease symptoms include metabolic bone disease, malnutrition, iron-deficient anemia, chronic diarrhea, abdominal bloating and distention, weight loss, damage to the jejuna mucosa and others (13). Conventionally, anemic patients are generally examined for CD. Typical asymptomatic patients with iron deficiency anemia have been evaluated by serological testing and have been found to exhibit a prevalence of CD ranging from 2.3 to 5.0% (14), and in some cases even between 10.3 to 15% (15). Further research has also indicated the prevalence of CD among premenopausal women with iron deficiency anemia (16). It has also been associated with liver disease, hypertransaminasemia and with a high risk of neuro-psychiatric disorders, such as peripheral neuropathy, mood swings, psychosis and epilepsy (17).

Gluten is rich in proline and glutamine residues, and therefore escapes proteolysis by human digestive enzymes, which lack post-prolyl endopeptidase activity. Thus, partially digested gluten peptides are deposited in the intestinal epithelial lumen and thus increase permeability by binding to CXCR3 receptors, and enter the lumen (18). Upon this entry process, the peptides undergo deamidation at glutamine residues by tissue transglutaminase (tTG) in the lamina propria region. The deamidated peptides bind to HLA-DQ2/DQ8 molecules with a greater affinity to gluten reactive $CD4^+$ T cells, activating the immune response (18,19). Notably, although one third of the Western population carries these HLA alleles (20), fortunately, only 3% develops CD, indicating that HLA-DQ2/DQ8 is necessary, but not sufficient for the development of the disease (4). Noticeably, 90 to 95% of individuals with CD express HLA-DQ2, and only 5 to 10% express HLA-DQ8 (19).

Although, the significant link between CD and HLA-DQ2/DQ8 has been established, CD is not present at the time of birth or before the consumption of gluten in diet (4) and generally does not appear before the age of 2 years, even in individuals expressing HLA-DQ2/DQ8 (21,22). In recent years, the prevalence of CD seems to have doubled and has been attributed to the environment, such as the administration of a gluten-rich diet to infants and the occurrence of certain gastrointestinal infections and immunological factors. It has been reported that breast-feeding for a long period of time and the delay in the ingestion of gluten-based food can perhaps postpone the onset of CD, particularly in young children (8). The occurrence of CD among children aged up to 4 months has not been observed upon the consumption of a gluten-rich diet; however, children at 7 months old have been found with autoimmunity (8,19).

3. Role of peptides in the development of celiac disease

The development of CD may be attributed to HLA. Two peptides that present HLA-DQ molecules, DQ (α1*0501, β1*02)/DQ2 or sometimes often DQ (α1*03, β1*0302)/DQ8, present on antigen-presenting cells (APCs), are the major genetic factors responsible for CD. HLA-DQ2, a heterodimer, found in 90% of patients with CD, is generally expressed in a *cis/trans* form (23). However, DR3 haplotypes exist in *cis* form; where HLA-DQ alleles HLA-DQA1*05 encode α chain and HLA-DQB1*0201 encodes a β chain of the dimer. A1-B8-DR3 is a classical Caucasian haplotype, whereas A26-B8-DR3 and Ax-B21-DR3 are typical haplotypes of CD in India (24). In *trans* form, DR7 haplotypes are related to the DQB1*0202 allele which encodes the β chain. The DR11, DR12 or DR 13 haplotype with the HLA-DQA1*05 allele on other chromosomes encode the α chain, and the α and β chains then unite to form CD-associated dimer. The α and β chains are encoded by HLA-DQA1*03 and HLA-DQB1*0302, respectively, in the case of HLA-DQ8 (25). In *cis* form, the α and β chains of DQ2 have been shown to be expressed by the HLA DQA1*0501 and HLA DQB1*0201 alleles of the DR3 haplotype. However, in *trans* form, the HLA DQA1*0501 allele of the DR5 haplotype encodes the α chain and DR7 haplotype with the DQB1*0202 allele on other chromosomes that encodes the β chain. Furthermore, these two chains are united to form the HLA DQ2 molecule on APCs (18).

Role of gluten and tTG in CD. Glutenin and gliadin are major protein fractions of wheat gluten. Gliadin fractions are more immunogenic than glutenin as they have more glutamine and proline residues. In addition, glutenin peptide epitopes are capable of activating DQ8-restricted T cell proliferation with QGYYPTSPQQS residues (26). Based on these amino acid sequences, are gliadins grouped into α, γ and ω. These gliadins contain epitopes that exhibit an intense affinity

towards DQ2/DQ8 molecules on APCs and are selectively accepted by gliadin-reactive T cells, which are only observed in the intestinal mucosa of CD-prone individuals (27). Three gliadin-derived DQ2-restricted epitopes, such as DQ2-α-I-gliadin, DQ2-α-II-gliadin and DQ2-γ-I-gliadin, and 2 DQ8-restricted epitopes, DQ8-α-I-gliadin and DQ8-I-glutenin, are recognized by gut T cells (26,28).

Gluten peptides become resistant to gastric, pancreatic and intestinal protease activity due to the high proline content and therefore enhance their retention in the small intestine. Furthermore, through epithelial transcytosis otherwise increases epithelial tight junction permeability, these gluten peptides reach the lamina propria and stimulate the tTG-mediated deamidation process (11,29). In this context, tTG catalyzes selective crosslinking or the deamidation of protein-bound specific glutamine residues; in addition, acidic pH in the stomach leads to the random deamidation of a number of peptides (29).

When genetically susceptible individuals ingest proline-rich gluten, the generation of gluten peptides occurs. These gluten peptides are not catalyzed by proteases and enter the lamina propria to form the crosslinking tTG that leads to deamidation. Deamidated peptides contain more immunostimulatory epitopes and are presented to gluten-reactive CD4 T-cells by HLA-DQ2/DQ8. Subsequently, these activated T-cells produce autoantibodies and other immunological mediators, which may lead to tissue damage [increased permeability, the dysfunction of intestinal tight junctions, infiltration of intraepithelial lymphocytes (IELs), the flattening of villi, and inflammation and malabsorption, as in late phase of the pathogenesis of CD] (18).

Fate of deamidated peptides and HLA-DQ2/DQ8. Deamidated gluten peptides with more negatively charged residues bind to HLA-DQ2 or HLA-DQ8 molecules with a high intensity. T cells that recognize the majority of DQ2-specific gliadin epitopes are tTG-targeted residues (26). A higher amount of glutamine and proline present in glutenins and gliadins of wheat gluten function as ideal substrates for TG2. The conversion of glutamine to glutamic acid residues through the deamidation process perhaps leads to a relatively large number of negatively charged residues of gliadin peptides. However, the affinity between gliadin epitopes and the peptide binding motif of HLA DQ2/DQ8 is crucial and leads to T cell proliferation (18,19). In corroboration, deamidated peptide-specific T cell proliferation has been clearly observed when T cells are mixed with deamidated peptide and incubated with monocyte-derived dendritic cells (30).

Moreover, T cells trigger the humoral-mediated immune response (HMIR) and thus stimulate B cells to produce corresponding antibodies for gluten peptides and tTG, and also produce groups of cytokines such as interferon (INF)-γ, interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6 and IL-8 at increased levels (31). These cytokines promote the development of enteric lymphocytes as cytotoxic cells and result in local inflammation. However, gluten-induced T cells trigger the immune system, affecting the synthesis of suitable immune components and decrease the production of IL-17 and IL-22, resulting in an adverse or abnormal mucosal structure. However, a gluten-reduced or -free food intake augments the secretion of IL-17 and IL-22 with better mucosal composition (32). Molecular approaches open up numerous strategies for the effective treatment of CD (19).

4. Diagnosis of celiac disease

The diagnostic criteria for CD from the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) were published during the 1990s (33). According to these criteria, the diagnosis was based on the morphological assessment of the small intestinal mucosa obtained at 3 distinct conditions, namely: i) Initial flat mucosa when the patient has ingested gluten; ii) improvement in the small intestinal mucosa upon the withdrawal of gluten from the diet; iii) deterioration of the mucosa with gluten challenge. In 1990, the criteria were revised for both childhood and adult CD when an individual is on a gluten diet, based on a small intestinal biopsy and typical histopathological morphology (34 and refs. therein). Furthermore, upon the complete restriction on gluten from the diet, there should be a full clinical response. However serological and biopsy analysis are gold standard tests (17). Histopathologically, CD presents a range in severity. Based on the severity of intestinal mucosal damage, several scoring systems for histological evaluation have been suggested (34).

Furthermore, antibodies, such as endomysial antibodies (EMA), tTG antibodies (tTGA) and antibiodies against gliadin (AGA) of the IgA-class are also significant diagnostic tools for CD, among which EMA and tTGA are widely used. There is a high occurrence of CD among individuals with IgA deficiency (8%). In addition, antibodies against gliadin can be measured by enzyme linked immunosorbent assay (ELISA) (15). Endomysium is a connective tissue protein found in the collagenous matrix of mucosal cells. Antibodies to endomysium can be measured using an immunochemical assay with monkey esophagus or human umbilical cord as a substrate in the diagnosis of CD. However, this test is costly and requires skilled personnel to perform. On the contrary, the measurement of tTGA using ELISA with guinea pig liver or human recombinant tTG as a substrate is less costly and more feasible than EMA (15).

The critical role of CD in the pathogenesis of CD is played by the HLA system; in particular, the role of HLA-DQ2/DQ8 in the development of CD has been well documented. As HLA-DQ2/DQ8 are heterodimers, in the majority of cases, carriers do not develop CD (40%) and therefore, genetic tests for the diagnosis of CD have limited applications (19), but can be used to rectify the uncertain diagnosis.

5. Possible therapies for celiac disease

Non-dietary strategies. A number of non-dietary strategies, that include decreasing intestinal tight junction (TJ) permeability using TJ regulators, such as larazotide acetate, the inhibition of tTG activity, the use of corticosteroids, such as budesonide, and altering the structure of gliadin using sequestering polymers are preferred for the treatment of CD (35,36). Such innovative options pave the way for alternative or adjunctive therapy. However, the effectiveness of this strategy perhaps is uncertain in terms of safety, efficacy and longer duration, rendering monitoring difficult. Furthermore, the

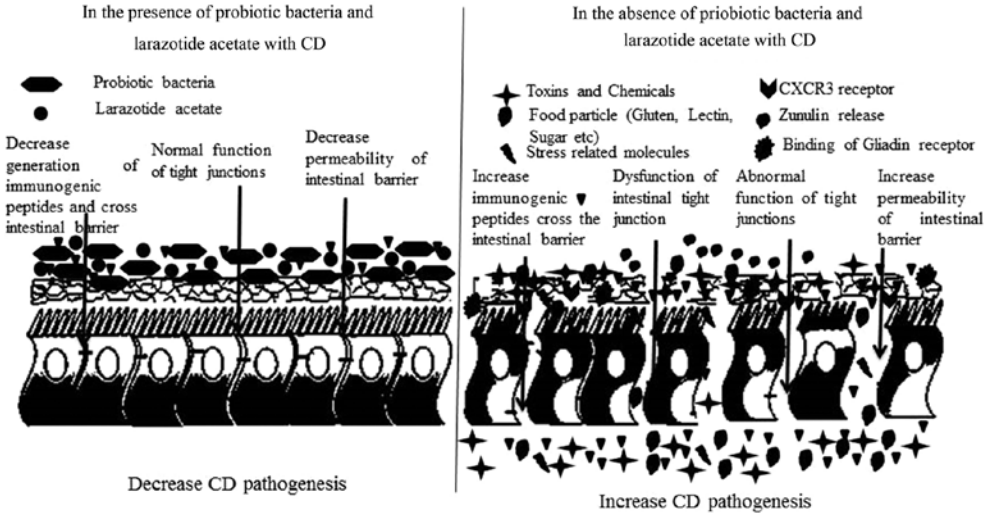

Figure 1. Effects of larazotide acetate on intestinal permeability (18).

follow-up of patients with CD with these therapeutic measures did not appear feasible (18). Intestinal TJ dysfunction leads to the increased permeability of intestinal barriers to gliadin peptides and exposes submucosal cells to immunogenic peptide-induced toxic effects. Larazotide acetate, a peptide regulating TJs, prevents the opening of intestinal epithelial TJs and has exhibited no severe adverse effects in clinical trials (Fig. 1) (36). The oral administration of this drug prior to each gluten intake perhaps helps to include the regulation of gluten-based food, alleviating the uncomfortable symptoms of CD. This perhaps requires further validation for the improvement of the efficacy of the drug to reduce gastrointestinal symptoms.

tTG is one of the endomysial auto-antigens which block the catalytic activity against auto-immunization and reduces the deamidation of glutamine-rich content. In an *in vitro* and *in situ* study, IgG and IgA classes of antibodies from patients with CD inhibited tTG activity in a dose-dependent manner (35). By contrast, tTG activities are inhibited by anti-tTG antibodies, and when targeted to active sites with relatively higher concentrations, residual enzyme activity would be sufficient to induce pathogenesis (37). Budesonide is one of the corticosteroid drugs which is often used to reduce looseness of the bowels, inflammation and intestinal tissue damage. Furthermore, budesonide effectiveness was previously assessed for the treatment of adults with CD; a group of patients with malabsorption was administered a only gluten-free diet only and the other group was administered a gluten-free diet with budesonide on a daily basis; after 28 days, the subjects treated with both the gluten-free diet and budesonide exhibited better health compared to the group that was treated only with the gluten-free diet (35). Furthermore, Goerres *et al* (38) reported that the combination of immunomodulatory medications, such as the steroids, azathioprine and prednisone, can be used with corticosteroids for the treatment of a number of autoimmune diseases associated with CD. In addition, polyhydroxy ethyl methacrylate-co-styrene sodium sulfonate [P(HEMA-co-SS)] is a sequestering polymer (non-absorbable). This polymer at gastric pH 1.2 and at intestinal pH 6.8 reacts with α gliadin peptide and induces changes in configuration and thus forms larger complex particles; therefore, tTG fails to recognize structurally altered peptides. Sequestering the gliadins with the polymer prevents the enzymatic action and the progression of disease halts (36). Instead, it has been proposed that this polymer-sequestered peptide would be discharged from the body prior to entering the blood (18,39).

Dietary strategies. Dietary therapy opted for the treatment of CD should be safe, effective and feasible with marginal or without side-effects. Nutritional dietary therapy involves a diet devoid of wheat, rye and barley. The presence of gluten in food could be reduced by biotechnological strategies using proteases produced by microbial cells, that hydrolyze immunogenic gluten peptides (40,41).

These ideal strategies perhaps offer a potential alternative or adjunctive treatment options; however, they raise important questions of safety, efficacy and monitoring during long-term treatment. However, gluten-free dietary therapy has been found to be safe, and has become the mainstay of CD management.

Gluten-reduced diet. It is possible to reduce the gluten content by producing less immunogenic varieties of wheat or related crop and or other biotechnological approaches to hydrolyze immunogenic gluten peptides using microbial proteases. Several studies have revealed that the follow-up of a strict gluten-free diet reduces the pathogenicity of CD (8). However, this type of diet is difficult to adhere to by patients, as it requires the lifetime exclusion of gluten-rich food from their regular diet. In addition, the FAO/WHO-recommended standards specify that the quality factors for gluten-free foods should not exceed 20 mg/kg. This consists of foods with ingredients from wheat (*Triticum* species), rye, barley, oats or other varieties. 'Gluten-free' foodstuffs substitute the original foodstuffs with the replacement of important basic nutrients with the same content of vitamins and minerals, and are produced under Good Manufacturing Practices (GMP) to avoid cross-contamination with prolamines. For this reason, patients with CD following a gluten-free diet, are recommended to take regular vitamin supplementation. Gluten-free fruits, vegetables and other food stuffs would also be consumed as a vitamin source or for micro nutrients. However, the availability

of gluten-free food products according to dietary guidelines is limited in developing countries, and when available, these products are costly. Therefore, it is of utmost importance to develop gluten-reduced wheat foods using microorganisms. Moreover, the *in vivo* efficacy of microbial proteases or enzyme preparations, systematic delivery against gastric acid pH in the stomach, formulation and dosage are the main challenges that need to be met (42). Over the past decade, research in probiotic lactic acid bacteria has proven to be efficient in the treatment of metabolic disorders and several types of cancer (40,43,44).

6. Importance of microorganisms in the treatment of celiac disease

The gut microflora has recently attracted attention again due to its critical role in health management and new concepts have been put forth regarding this by medical researchers (45). Consequently, the association between human health and the gut microbiota is significantly acknowledged and confirms that a healthy gut microflora is crucial for the comprehensive health of an individual (46). Over the period of host-microbial co-evolution, the intestine adjusts to bidirectional host-microbial exchange and also harbors a diverse microbiota which is separated by a single layer of epithelial cells. The interaction of the gut with its commensal microorganisms plays a crucial role in promoting homeostatic functions, such as immunomodulation, the upregulation of cytoprotective genes, the prevention and regulation of apoptosis, and the maintenance of barrier function, among others (46).

Several studies have reported that, at the epithelial level, a number of factors, such as the masking or modification of microbial-associated molecular patterns (MAPS) that are generally recognized by host receptors, as well as the inhibition of the NF-κB inflammatory pathway allow host cells to tolerate commensal microorganisms (47). Furthermore, some gut bacteria produce anti-inflammatory compounds, which result in a controlled inflammatory response, conferring protection against pathogens. Sometimes, the generation of a low-grade inflammatory response from commensal bacteria could be realized to boost the immune system against the pathogen (48). In addition, some gut bacteria produce a variety of metabolites ranging from relatively non-specific fatty acids, proteases with antimicrobial property and peroxides to highly specific bacteriocins (49). The gut microbiota, through these and other related mechanisms, have been found to play a crucial role in protecting the host from invading pathogens. These observations suggest that increasing the number of beneficial bacteria in the gut may be helpful in maintaining gut health and this could be achieved by the application of probiotics (19).

Supplementation of probiotics as an alternative treatment for CD. Microbiologists in the late 18th century identified that the gut microbiome of healthy individuals differed from that of infected individuals. The beneficial microorganisms found in the gut were termed as probiotics. The term probiotic means 'for life' and it currently refers to the beneficial effects on humans and animals. As per FAO/WHO (2001; http://www.fao.org/3/a-a0512e.pdf), probiotics were defined as living bacteria and when administered in an adequate quantity, confer health benefits to the host. However, the history of probiotics dates back to late 18th century. The credit for first observation made on the positive role of some selected bacteria was attributed to Eli Metchnikoff (1908). According to their findings, the bacterial community inhabiting the large intestine of humans was a source of toxic substances that were detrimental to the host, intoxicating the blood and contributing to the ageing process, leading to autointoxication (50).

In 1907, Metchnikoff had postulated that the natural fermentation of milk by lactic acid producing bacteria, i.e., *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, prevented the growth of proteolytic species and therefore, lactic acid bacteria were used for the implantation of beneficial microbiota in the gastrointestinal tract. Furthermore, Fuller's (51) definition for probiotics reads as 'live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance'. Subsequently, Havenaar (52) corresponded to the probiotic definition with the following description: A viable mono or mixed culture of microorganisms which, applied to animals or man, beneficially affects the host by improving the properties of the indigenous microflora. At the end of the millennium, Salminen *et al* (53) proposed the following definition: 'Probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of host'.

The probiotic microorganisms consist mostly of the strains of the genera *Lactobacillus* and *Bifidobacterium*, *Bacillus*, *Pediococcus* and others (54). Of note, the probiotic effect is strain-specific; i.e., different strains of the same species are always unique. Furthermore, they may differ in their adherence sites (site-specific) and may also exert specific immunological effects. Consequently, their action on a healthy and an inflamed mucosal milieu differs (55). Proposed mechanisms of probiosis include effects on the composition and function of the intestinal microbiome. Probiotics produce antimicrobial agents or metabolic compounds that suppress the growth of other microorganisms or compete for receptors and binding sites with other intestinal microorganisms on the intestinal mucosa (56), and thereby prevent the pathogen colonization. Probiotics strengthens the intestinal barrier, which may result in the maintenance of immune tolerance, and the decreased translocation of bacteria across the intestinal mucosa.

In addition, probiotics can modulate intestinal immunity and alter the responsiveness of the intestinal epithelia and immune cells to microorganisms in the intestinal lumen (57). Furthermore, the regulation of apoptosis and inflammatory action, the inhibition of procarcinogenic enzyme activity, and the induction of enzymatic activity that aids digestion and nutrient absorption enhances host health. These probiotic functions are the result of very complex mechanisms of consortia of microorganisms (58). Probiotics can also be found in dairy and non-dairy products. Probiotics are administered for the prevention and management of several diseases and disorders that mainly include traveler's diarrhea, rotavirus gastroenteritis, pouchitis, vaginosis, cirrhosis, hyperlipidemia, *Helicobacter pylori* infection, colitis, acute and chronic gastroenteritis, irritable bowel syndrome, inflammatory bowel disease and neonatal enterocolitis (59). In addition, maldigestion-related conditions, such as lactose intolerance, cow's milk protein allergy, soy protein allergy and gluten intolerance can also be treated and managed using probiotics (19).

Detoxification of gluten peptides by probiotics and their proteases. There are two alternative gluten peptide hydrolysis strategies as follows: i) The medical approach which the hydrolyzation of immunogenic gluten peptides following ingestion in the gastrointestinal tract; and ii) the food technological approach which involves the to hydrolyzation of gluten peptide prior to the gluten ingestion during food processing (60).

i) *Medical approach*. Gluten degradation can be performed by prolyl endopeptidases of microbial sources that lend themselves to large-scale production (61). Prolyl endopeptidases (PEP), an endoproteolytic enzyme of microbial origin, can readily cleave proline-rich gluten epitopes in contrast to human digestive enzymes (62). Several researchers have reported the use of probiotics or their enzymes for gluten reduction in wheat foods. For example, on long-term wheat flour fermentation, VSL#3 probiotic bacterial preparation has been shown to effectively reduce gluten toxicity; surprisingly, no increase in the infiltration of $CD3^+$ intraepithelial lymphocytes was observed and moreover, a reduced zonulin release was observed when the jejuna of patients with CD was exposed to peptic-tryptic digest from VSL#3 (40). Gluten-detoxifying gelatin-encapsulated capsules of *Myxococcus xanthus* prolyl endopeptidase (MX PEP) were characterized and developed to protect the gastric environment, with safe release into the duodenal region and a reduction in gluten-induced inflammation (41). An *in vivo* study reported that following the ingestion of gluten pre-treated with ALV003 to patients with CD, the gluten-specific T cell response was reduced compared to the placebo group (63). These studies clearly portrayed that microbial or probiotic proteases perhaps, used as an efficient tool to combat CD either by or other means.

Numerous studies have revealed the ability of prolyl endopeptidase from *Flavobacterium meningosepticum* in hydrolyzing the 33-mer gliadin peptide. Shan *et al* (64) also recommended the use of this enzyme for oral therapy for patients with CD. Furthermore, these findings were supported by an *in vivo* study on rats, where PEP perfusion together with gluten peptides into the rat intestine accelerated the digestion of gluten by approximately 50 to 100% (61). In addition, Pyle *et al* (65) reported that the pre-treatment of gluten with PEP from *F. meningosepticum*, prevented the development of fat and carbohydrate malabsorption. PEP from *Myxococcus xanthus*, *Sphingomonas capsulata* (41,64) and *Lactobacillus helveticus* (66), *Bacillus* sp. (67,68) also supported gluten detoxification properties. However, Shan *et al* (64) mentioned that PEP were inactivated by pepsin and acidic conditions in the host stomach. Similarly, results were reported by Stepniak *et al* (69) for *Aspergillus niger*. This enzyme can be produced on a large scale at food-grade quality in industries at a reasonable cost and can be used as an oral supplementation in patients with CD to reduce the burden of ingested gluten (70).

ii) *Food technological approach*. Proteolysis by sourdough starter culture usage has become a novel approach with which to reduce gluten toxicity during food processing for patients with CD (71,72). In several studies, wheat flour fermentation with lactobacilli has been shown to decrease the CD-inducing effect of gluten (72). This observation has been extrapolated by Di Cagno *et al* (73) to produce sourdoughs that contain 30% of wheat flour and the remaining 70% of non-gluten flour with selected lactobacilli. The mixed starter (*Lactobacillus alimentarius*, *L. brevis, L. sanfranciscensis* and *L. hilgardii*), was able to hydrolyze gliadin fractions and the bread prepared from that sourdough was tolerated by patients with CD, which was proven by an intestinal permeability challenge. Furthermore, Di cagno *et al* (73) followed the same approach for preparing pasta for patients with CD. In another study, the combination of *Lactobacillus alimentarius*, *L. brevis*, *L. sanfranciscensis* and *L. hilgardii* were used as starter culture for pre-fermenting durum wheat semolina. The dough was then freeze-dried and mixed with buck wheat flour at a ratio of 3:7 and the pasta was prepared. The immunological assay of this sample has shown that the concentration of gluten has decreased from 6,280 to 1,045 ppm. However, this level of gluten was higher than the threshold levels as per the Codex Alimentarius Commission of WHO. According to Codex Alimentarius, foods containing gluten <20 ppm can be labeled as 'gluten-free', while products containing gluten >20 and up to 100 ppm can be labeled as 'gluten-reduced'. However, the combination of lactobacilli with two fungal proteases from *Aspergillus niger* and *A. oryzae* has decreased the gluten concentration of wheat flour below 10 ppm during fermentation (74).

Corroborating this, Gobbetti *et al* (72) observed that functional probiotics contribute to food tolerance through their array of enzymes. The probiotic VSL#3 preparation containing *Streptococcus thermophilus*, *L. plantarum*, *L. casei*, *L. delbrueckii spp. bulgaricus*, *Bifidobacterium breve*, *B. longum* and *B. infantis* were the starters in baking. During fermentation, there was a marked degradation of wheat gluten (12). Upon the exposure of peptic-tryptic digest of VSL#3 fermented dough to celiac jejunal biopsies, there was no increase in the infiltration of $CD3^+$ intraepithelial lymphocytes (40). In the food industry, to improve the quality and other food parameters, microbial transglutaminases (mTG) are being used (75). The mTG formulated food could able to generate T cell reactive gluten epitopes by deamidation. Therefore, it is recommended not to use mTG in food formulations for celiacs. Based on these functions, it has been hypothesized that probiotics are distinctly involved in the dietetic management of CD (76).

7. Mechanisms of action of probiotics in celiac disease

Strengthening of intestinal epithelial barrier by probiotics. The intestinal epithelium is in constant interaction with the luminal contents, as well as the enteric microbiota. The major function of the intestinal epithelium is the maintenance of epithelial integrity. Generally, the intestinal mucous layer, antimicrobial peptides, secretory IgA-a dimer antibody, and the epithelial junction adhesion complex constitutes the defense system of the intestinal barrier (77). The disruption of this barrier facilitates the invasion of bacterial and food antigens into the submucosa, which further induces inflammatory responses, resulting in intestinal disorders, such as inflammatory bowel disease (IBD) (78). Furthermore, probiotic bacteria have been extensively studied for their beneficial role in the maintenance of the intestinal epithelial barrier. It has been reported that probiotics enhance the expression of genes that participate in tight junction signaling and possibly thereby reinforce the intestinal barrier integrity (79). Lactic acid

bacteria (LAB) modulate the regulation of genes encoding adherence junction proteins in a T84 cell barrier model, which include E-cadherin and β-catenin. The incubation of intestinal cells with LAB differentially enhances the phosphorylation of adherence junction proteins and results in the formation of protein kinase C (PKC) isoforms, thereby positively regulating epithelial barrier function (80).

Studies have demonstrated that probiotics mediate the restoration of the impaired barrier function. In addition to the prevention of the enteropathogenic Escherichia coli (EPEC)-mediated disruption of the mucosal barrier, *E. coli* also restores the mucosal integrity in T84 and Caco-2 cells. This effect was found to be mediated by the enhanced expression and redistribution of tight junction proteins of zonula occludens 2 (ZO-2) and PKCl, resulting in the reconstruction of the TJ complex (81). Moreover, *Lactobacillus casei* isolates have been shown to sustain intestinal barrier function through similar mechanisms; they have also been shown to protect the epithelial barrier and to increase tight junction protein expression through the activation of the p38 and extracellular regulated kinase signaling pathways in *in vivo* and *in vitro* experiments (82). These probiotics enhance the strength of the intestinal epithelial barrier indirectly; thus, they may prove to be an effective therapy for CD (19).

Adhesion to intestinal mucosa by probiotics. The ability to adhere to the intestinal mucosa is considered as one of the main selection criteria for potential probiotics as it prolongs their persistence in the intestine and thus allows the probiotics to exert their beneficial effects (83). Several probiotic bacterial surface proteins have also been proven to promote mucous adhesion. The majority of the probiotic bacterial species are Gram-positive strains consisting of a thick peptidoglycan layer, polysaccharides such as teichoic acid and lipoteichoic acid, and various cell-surface proteins, including S-layer proteins. These typical cell surface structures are in direct contact with the environment and may function as adhesion factors, antigens, or receptors. In addition, they are also known to take part in various physiological functions (47). Based on the wide variation of molecular structures, lactobacilli exhibit various adhesive properties on mucin and mucin carbohydrate chains. Based on these observations, it has been suggested that *Lactobacillus* adapt to the constantly changing intestinal environment of the host, and further indicate that the adhesion factors of *Lactobacillus*, exhibiting specific binding affinities, allow them to selectively colonize inside the host while concurrently avoiding competition with other bacteria. This process is mediated by a variety of proteins, saccharide moieties and lipoteichoic acids (84). *Lactobacillus reuteri* produced mucus-binding protein (MUB) is the most studied example of mucus-targeting bacterial adhesins (85). The proteins accounting for the mucous adhesion phenotype of probiotics are mainly secreted and are surface-associated proteins, which are either anchored to the membrane through a lipid moiety or are embedded in the cell wall. Under certain conditions, these MUB play a crucial role in promoting gut colonization through the degradation of colonocytes extracellular matrix and or by establishing close contact with the epithelium (86). In an example, MapA (mucous adhesion-promoting protein) mediates binding of few LAB, such as *L. reuteri* and *L. fermentum* to mucus. *L. plantarum*, has also been shown to induce MUC2 and MUC3 mucins and inhibited the adherence of EPEC (83).

Furthermore, VSL#3, a probiotic mixture, has been reported to enhance the expression of cell surface mucin genes (87). In addition, probiotics also lead to modifications in the intestinal mucins that prevent pathogen binding. Of note, the binding protein cleaved into an antimicrobial peptide confers an anti-pathogenic effect to the host, emphasizing the pleiotropic effect of probiotic surface proteins (88). Moreover, adhesion properties of gluten-hydrolyzing probiotics promise to improve the overall health of patients with CD (12).

Competitive exclusion of pathogens by probiotics. The concept of 'competitive exclusion' was first proposed by Greenberg (89), in which one particular bacterial species strongly competes for the receptor binding site in the intestinal tract with other species of bacteria. The mechanisms adapted by the bacterial species to exclude the competitive species vary and mainly include the establishment of a hostile environment, competing for available receptor sites, the production of antimicrobial compounds, and the competitive depletion of available nutrients. To elaborate further, in order to gain a competitive advantage, bacteria modify their environment unfavorable for the survival of their competitors by producing metabolites, such as organic acids (90). In general, probiotic cells are capable of inhibiting the attachment of pathogenic bacteria by means of steric hindrance at enterocyte pathogen receptors (88). Several studies have reported the effect of probiotics on the competitive exclusion of pathogens *in vitro* as well as *in vivo*. In the case of *Lactobacillus rhamnosus*, it was found to eliminate herpes simplex virus type I by activating macrophages (91). In addition, probiotics in the gut may also help to eradicate *Helicobacter pylori*, mitigating the frequency of epigastric pain, vomiting, nausea and diarrhea (92).

Effect of antimicrobial compounds produced from probiotics. The production of low molecular weight compounds (<1,000 kDa), such as organic acids, and antibacterial substances called bacteriocins (>1,000 kDa) are majorly responsible for the health benefits conferred by probiotics in the host (93). The organic acids produced by probiotics mainly lactic and acetic acids exert potent inhibitory effects on Gram-negative bacteria. These organic acids enter the bacterial cells in their un-dissociated form and in the cytoplasm they undergo dissociation. Furthermore, the accumulation of ionized form of organic acids and/or the lowering of the intracellular pH results in the death of target bacteria (83). A number of lactic acid bacteria produce bacteriocins, such as lactacin B (*L. acidophilus*), plantaricin (*L. plantarum*) and nisin (*Lactococcus lactis*) (94). Bacteriocins destruct the target cells by forming pores or inhibiting the cell wall synthesis. To consider, nisin forms a complex with lipid II and thereby inhibit the biosynthesis of cell wall (95). Bacteriocin production perhaps encourages the establishment and increases the prevalence of bacteriocin-producing strains, thus directly inhibiting pathogens in the gastrointestinal tract (12).

Furthermore, probiotics producing anti-metabolic substances inhibit the growth of fungi and other species of bacteria (96). Furthermore, the production of antifungal substances, such as

Table I. List of commonly available commercial probiotics for human consumption (110,111).

Microorganism	Company	Microorganism	Company
Bifidobacterium adolescentis ATCC 15703	Chr. Hansen	*Bacillus cereus* strain IP 5832 (ATCC 14893)	Marion Merrell Dow Laboratories
Bifidobacterium animalis Bb-12	Chr. Hansen	*Bacillus subtilis*	Tendiphar Corporation
Bifidobacterium essencis	Danone® (Activia)	*B. subtilis*	Bidiphar-BinhDinh Pharmaceutical
Bifidobacterium infantis	Yakult Danone®	*B. subtilis* 2335 and *B. licheniformis* 2336	Biofarm
Bifidobacterium lactis	DSM	*B. subtilis* and *Lactobacillus acidophilus*	IVAC
Bacillus lactis DR10	Danisco (Howaru™)	*B. pumilus*	Biophar Company
Lactobacillus acidophilus	Chr. Hansen	*B. cereus* strain GM	Geyer Medicamentos S.
LA-1/LA-5	Rhodia		
NCFM	Nebraska Cultures		
DDS-1	Snow Brand Milk Products		
SBT-2062			
Lactobacillus casei	Yakult (Yakult®), Danone®	*B. polyfermenticus* SCD	Binex Co., Ltd.
Lactobacillus fermentum RC-14	Urex Biotech	*B. subtilis* strain RO179 *Enterococcus faecium*	Hanmi Pharmaceutical Co., Ltd.
Lactobacillus lactis L1A	Essum AB	*B. subtilis*, *B. polymyxa*, *B. pumilus* *B. laterosporus*	Nature's First Law
Lactobacillus rhamnosus	Valio	*B. subtilis*	Pasteur Institute of Ho
GG	Urex Biotech		Chi Minh City
GR-1	Essum AB		
LB21	Probi AB		
271			
Lactobacillus plantarum 299v Lp01	Probi AB	*B. cereus*	Mekophar, Pharmaceutical Factory

benzoic acid, methylhydantoin, mevalonolactone and short-chain fatty acids by *Lactobacillus* spp. are quite evident (97). Another study reported the production of proteinaceous compounds exhibiting antifungal properties by *Lactobacillus coryniformis* (98). In addition, Dal Bello *et al* (99) identified and chemically characterized the 4 antifungal substances produced by *L. plantarum* FST 1.7, including lactic acid, phenyl lactic acid and 2 cyclic dipeptides, [cyclo(L-Leu-L-Pro) and cyclo(L-Phe-L-Pro)]. A similar study reported the production of the antifungal cyclic dipeptides, cyclo (L-Phe-L-Pro) and cyclo (L-Phe-traps-4-OH-L-Pro), by lactic acid bacteria, which inhibited the growth of food borne fungi (100).

Immunomodulatory effect of probiotics. Probiotics interact with intestinal epithelial cells, macrophages, lymphocytes and dendritic cells (101). In the adaptive immune response, B and T cells specific for pathogens play an important role, while the innate immune system responds to pathogen-associated molecular patterns (PAMPs) which are shared by the majority of pathogens. The pattern recognition receptors (PRP) that bind to PAMP trigger primary immune response to pathogens (47). Furthermore, Toll-like receptors (TLRs) are transmembrane proteins that are expressed on various immune, as well as non-immune cells. In humans, there are 11 classes of TLRs that have been identified thus far. Amongst these, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are associated with the outer membrane and primarily respond to bacterial surface-associated PAMPs. On the other hand, TLR3, TLR7, TLR8 and TLR9 are found on the surface of endosomes, and they respond primarily to nucleic acid-based PAMPs from viruses and other bacteria (101). In addition, the TLR-mediated signaling has been shown to regulate the maturation of dendritic cells, upregulating various maturation markers such as CD80, CD83 and CD86, as well as CCR7 chemokine receptor. Moreover, it has been observed that following activation by commensal and probiotic microorganisms, dendritic cells initiate an appropriate response, such as the differentiation of Th_0 to T_{reg}, which exhibit an inhibitory effect on the Th_1, Th_2 and Th_{17} inflammatory response (102).

Probiotics reduce intestinal inflammation via the down-regulation of TLR expression. The secretary metabolites from probiotics prevent the entry of TNF-α into blood mononuclear cells and also arrest NF-κB signaling in enterocytes (101). Furthermore, TLRs recognize peptidoglycan, a major component of Gram-positive bacteria. Several studies have demonstrated the necessity of TLRs for lactobacilli to exert their immunomodulatory effects. It has been shown that the cell wall components of lactobacilli mainly diacylated membrane anchors of lipoproteins and lipoteichoic acids take part in signaling upon concurrent binding to TLR2 and TLR6. The activation of

TLR2 enhances the production of cytokines and also increases transepithelial resistance to invading pathogens (12,103). This evidence supports immunomodulatory functions of probiotic bacteria in their host and reduce the CD with managing the inflammation inducing transmembrane signals (19,104).

Safety and efficacy of probiotics for human use. It is mandatory that any given probiotic strain should not be at any significant risk with regard to transferable antibiotic resistance (105). Furthermore, if any strain is under evaluation belonging to a particular species, it needs to be examined for toxin production and/or hemolytic activity (106). The assessment of a lack of infectivity by a probiotic strain in immune compromised individuals would be an additional advantage to human use (107). The outcome of efficacy studies on probiotics are required to be proven significantly with benefits in human trials, such as an improvement in conditions, symptoms, signs, wellbeing/quality of life, a reduced risk of disease or longer time to next occurrence or faster recovery from illness (19,104). Each of the parameters should have a proven association with probiotics and may be helpful for CD therapy (108).

8. Commonly available commercial probiotics

The indigenous microbiota of infants is dominated by bifidobacteria, which are recognized shortly after birth. Their proliferation is stimulated by the glycoprotein components of k-casein in human colostrum and, to a lesser extent, human milk. The extent of bifidobacterial population decreases with the increasing age of the human subject and eventually becomes the third most abundant genus (accounting for approximately 25% of the total adult gut microbiota) after the genera *Bacteroides* and *Eubacterium* (109). The commonly available probiotics are from the strains belonging to the genera, *Lactobacillus*, *Streptococcus*, *Bifidobacterium* and *Bacillus* spp. The commercially available probiotic products in the market (110,111) are listed in Table I. Bifidobacteria are microorganisms of paramount importance in the active and complex ecosystem of the intestinal tract of humans and human gastrointestinal and genitourinary tracts, the exact ratio of which is determined mainly by age and diet (19).

Bacillus spp. have been used as a probiotic for at least 50 years in an Italian product commercialized as Enterogermina® ($2X10^9$ spores). Among this group, some species that have been evaluated for their probiotic potential, which include *Bacillus subtilis*, *Bacillus clausii, Bacillus cereus, Bacillus coagulans* and *Bacillus licheniformis* (112). Currently, in the market, probiotic products containing *GRAS* isolates of *Bacillus* are increasingly available, including *Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, Bacillus pumilus, Bacillus clausii* and *Bacillus coagulans* (111). The members of *Bacillus* have been proven to form dormant forms as endospores, a protective mechanism to overcome the unfavorable conditions, such as nutrient deprivation and other factors of environmental stress. The tough coat of endospores confers resistance to high temperatures, low pH and low moisture conditions (67,68,113). Taking into account the advantage of this property, unlike *Lactobacillus*, *Bifidobacteria* and other commonly used probiotic lactic acid bacteria, Bacillus probiotics can be used in the form of spores, which has an indefinite shelf life and does not require refrigeration (114). Furthermore, studies have demonstrated the potential probiotic attributes of the *Bacillus* spp. and their efficacy in gastrointestinal disorders (112,113). Consequently, *GRAS* isolates of *Bacillus* spp. have attracted the attention of the probiotic industry, having more advantages over conventional probiotic lactic acid bacteria.

Other probiotic bacteria include *Leuconostoc mesenteroides*, *Leuconostoc lactis*, *Streptococcus thermophilus*, and *Pediococcus* spp. (115). Among the probiotic yeasts, the most common genus, *Saccharomyces cerevisiae* has been used as a potential probiotic. The potential probiotic effect of *S. cerevisiae* and *S. cerevisiae* var. *boulardii* has been demonstrated since they are able to tolerate low pH and bile and protect against bacterial infections through the reduction of the intestinal pro-inflammatory response (116) and have been used worldwide as a therapeutic agent for diarrhea and other gastrointestinal disturbances caused by the administration of antimicrobial agents (117). Although lactic acid bacteria are beneficial in alleviating or managing various health issues, in recent times, these bacteria are used in alleviating a high intolerance of gluten allergy leading to CD (12,19,104); therefore, they have become a food of choice by patients with CD.

9. Conclusion

Current probiotic research aims at the use of the normal, healthy gut microbiota as a therapy for CD and their implications on human health. From all these aspects, probiotics are generally safe and cost-effective compared to the drugs available in the market. *Bifidobacterium* spp. and *Lactobacilli* spp. are the promising agents for probiotic therapy for patients with CD. Further studies should emphasize on microbiota characterization with potential benefits to gut health of patients with CD. The molecular mechanisms of probiotic action are in a tranquil state and require to be characterized. The future metabolomic approach would provide insight into the knowledge of the mechanisms of the microbiota for CD therapy.

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Authors' contributions

DG conceived the study, drafted the manuscript, and was also involved in editing, reviewing and revising the manuscript, and also communicated the manuscript to the journal. AR was involved in the conception of the study, as well as in the processing of the data acquired for this review, the drafting of the manuscript and processing of the figure. AR was also

involved in the study design and editing of the manuscript. Both authors have read and approved the final manuscript.

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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