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Review

Pro-Pre and Postbiotic in Celiac Disease

Mariangela Conte [1,2], Monia Porpora [1,2], Federica Nigro [3,4], Roberto Nigro [5], Andrea Luigi Budelli [3,5], M. Vittoria Barone [1,2,*] and Merlin Nanayakkara [1,2]

1 Department of Translational Medical Science (Section of Pediatrics), University of Naples Federico II, Via S. Pansini 5, 80131 Naples, Italy; maryconte_92@hotmail.it (M.C.); monia.porpora@hotmail.it (M.P.); merlinnanayakkara@gmail.com (M.N.)
2 European Laboratory for the Investigation of Food Induced Diseases (ELFID), University of Naples Federico II, Via S. Pansini 5, 80131 Naples, Italy
3 School of Engineering, Niccoló Cusano University, 00166 Rome, Italy; federica.nigro@itpna.it (F.N.); andrealuigi.budelli@unina.it (A.L.B.)
4 I.T.P. Innovation and Technology Provider S.r.l., Via Bisignano a Chiaia 68, 80121 Naples, Italy
5 DICMAPI, University of Naples Federico II, 80125 Naples, Italy; rnigro@unina.it
* Correspondence: mv.barone@unina.it; Tel.: +39-0817464568

Abstract: Celiac Disease (CD) is an autoimmune disease characterized by inflammation of the intestinal mucosa due to an immune response to wheat gliadins. It presents in subjects with genetic susceptibility (HLA-DQ2/DQ8 positivity and non-HLA genes) and under the influence of environmental triggers, such as viral infections and intestinal microbiota dysbiosis. The only treatment currently available in CD is a gluten-free diet for life. Despite this, the intestinal dysbiosis that is recorded in celiac subjects persists, even with adherence to dietary therapy. In this review, we have analyzed the literature over the past several decades, which have focused on the use of pro-, pre- and post-biotics in vitro and in vivo in CD. The study of probiotics and their products in CD could be interesting for observing their various effects on several different pathways, including anti-inflammatory properties.

Keywords: celiac disease; pro- pre- and post-biotic; inflammation

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1. Introduction

Celiac disease (CD) is a common systemic disease that primarily affects the small intestine [1], due to the abnormal response of the immune system to gluten ingestion. It happens in subjects with genetic susceptibility (HLA-DQ2/DQ8 positivity and non-HLA genes) and under the influence of environmental triggers, including, a part from gluten, viral infections and intestinal microbiota dysbiosis [2]. Although 30–40% of the world population carry the HLA DQ2/DQ8 genotype, only 1–1.5% of them express the CD phenotype, which means that other genes together with other environmental factors may take part [2,3]. Typically, the inflammation in CD includes an increased intraepithelial lymphocyte (IEL) count, most often >25/100 cells [4,5]. Other features of CD are the presence of an adaptive T-cell-mediated response to gluten [6,7] and of specific endomysial antibodies (EMA, also called AEA), anti-tissue transglutaminase antibodies (TTG, a-tTG, TTA), and/or deamidated antigliadin antibodies (DGP) that play an important role in the serological work-up for CD. These antibodies strongly support the diagnosis of CD. In children, the intestinal biopsy is necessary where the antibody title is low and symptoms are absent. In paediatric patients with high tTG-IgA, ESPGHAN recommends that the decision whether or not to perform duodenal biopsies should be made during a shared decision making process between the paediatric gastroenterologist/coeliac disease specialist, the parent(s)/carer(s), and if appropriate, the child and any way when patients are on a gluten-containing diet [8]. The diagnosis is confirmed by the finding of an intestinal athrophy with shortening of the intestinal villi and increase of the crypt proliferation. Gluten is

poorly digested in the human intestine with or without CD. Undigested gluten peptides cross intact the mucosa of the small intestine. In the submucosa of the small intestine the human enzyme transglutaminase 2 (tTG) deamidates gluten peptides, leading to an increase in the affinity binding to human leucocyte antigen (HLA) DQ2 and HLA DQ8 molecules, subsequently triggering an inflammatory reaction in patients with CD [7]. The only treatment currently available in CD is a gluten-free diet for life. Recently, in CD, a role for the gut microbiota, has been described. There are several cross-sectional studies comparing microbiota in patients and controls, which describe a dysbiosis in CD patients before, and after, the gluten free diet. The aim of this review is to describe the most recent literature on pro-, pre and post-biotic in CD (See Figure 1).

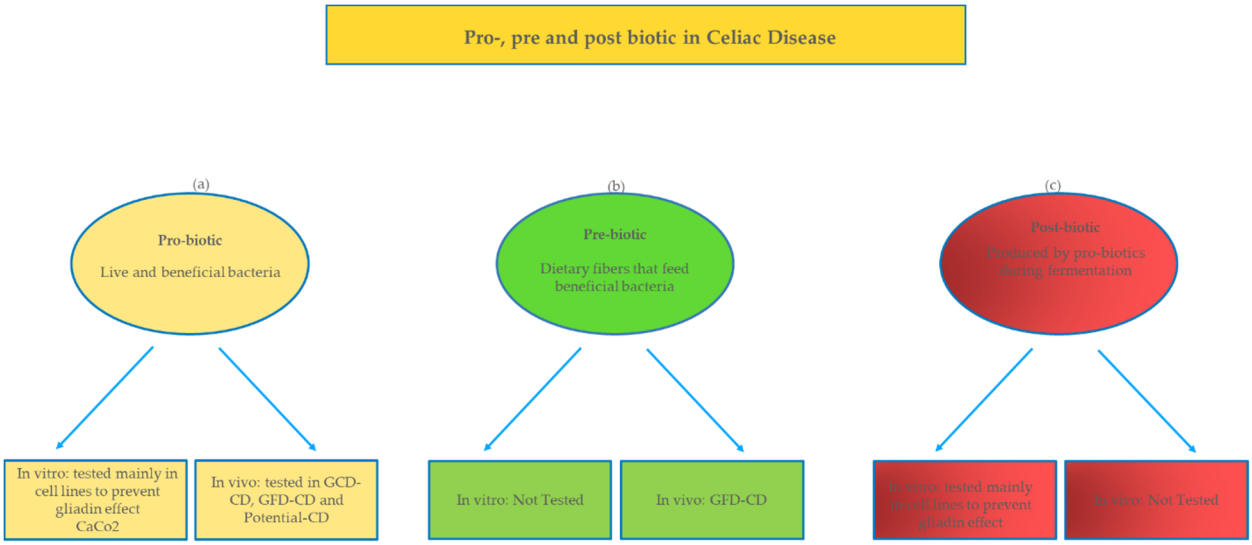


Figure 1. Schematic representation of the in vitro and in vivo experiments performed in CD with pro (**a**), pre (**b**) and post-biotics (**c**).

Pro-biotics are microorganisms, most of which are bacteria similar and/or the same to the beneficial bacteria that occur naturally in the human gut. Pro-biotics have been widely studied in a variety of gastrointestinal diseases. Pro-biotics have an important role in the maintenance of immunologic equilibrium in the gastrointestinal tract through the direct interaction with immune cells. Pro-biotic effectiveness can be species-, dose- and disease-specific, and the duration of therapy depends on the clinical indication.

Dietary fibers can be metabolized by microbes in the gastrointestinal tract. Human food enzymes are unable to digest most complex carbohydrates and plant polysaccharides. Instead, these polysaccharides are metabolized by microbes that generate short-chain fatty acids (SCFAs), including acetate, propionate and butyrate.

Post-biotics are known as "non-viable probiotics", "inactivate probiotics" or "ghost probiotics" and refer to both non-viable microbial cells and soluble factors secreted by live bacteria or released after their lysis, including various cell surface components, lactic acid, short-chain fatty acids (SCFAs) and bioactive peptides among other metabolites. When administrated in sufficient amounts, these may contribute to the improvement of host health, even though the exact mechanisms are not yet well-known. The bacterial inactivation usually occurs by a mild heat treatment. The advantages of using post-biotics, include their higher stability, as they do not contain living bacteria, and; their higher levels of safety compared to pro-biotics, as they reduce the risk of microbial translocation, infection or enhanced inflammatory responses in consumers with imbalanced or compromised immune systems.

2. Materials and Methods

Pub Med search of articles on "Celiac Disease and Pro-biotics", Celiac Disease and Pre-biotics", "Celiac Disease and Post-Biotics" have been done from these we have selected literature with 6/68 references from the last 20 years, 14/68 from the last 15 years, 18/68 references from the last 10 years and 30/68 from the last 5 years

3. Microbiota Is Altered in CD

The microbiota is the ecological community of microorganisms within a defined environment. It is concentrated in the intestinal tract and is rapidly altered by external factors. The microbiome is the collective genomes of all microorganisms from a given environmental niche. Changes in the microbiota and consequently in the microbiome, impact the homeostasis of the whole body. The composition of the gut microbiota depends on many factors, such as age, geographical position, diet, genetics, natural birth and breast-feeding, so it is impossible to have the same microbiota in everybody and consequently to define the ideal microbiota. The gut microbiota influences immunity system response and inflammation. The microbiota plays a fundamental role in induction, training, and functioning of the host immune system so its alteration is generally associated with the immunity system response and inflammation [9]. In CD, alterations of the microbiota have been found. Interestingly the microbiota of CD patients can change in the different stage of the disease: There are microbiota signatures different for GCD-CD (Gluten Containing Diet Celiac Disease) and GFD-CD (Gluten Free Diet-Celiac Disease) respect to non CD subjects.

3.1. Changes in the GCD-CD Microbiota Respect to Controls

Patients in the acute phase of the disease at GCD present alteration of the gut microbiomawith increasing *E. coli*, ML615J-28, *Slackia*, *Victivallaceae*, *Enterobacteriaceae*, *Clostridiaceae*, *Coriobacteriaceae* and unclassified specie of *Clostridiales* and *Lachnospriraceae*, decreasing *C. lituseburense*, *Lactobacillus*, *F. Prausnitzii*, *Bifidobacterium*, *Dorea*, *B. wexlerae*, *Lachnospriraceae*, *A. hadrus*, *E. hallii*, *Veillonellaceae*, *R. bromi*, *R. faecis* CD patients showed a gut dysbiosis [10]. In these patients the bacteria that have a protective effect such as *Bifidobacteria*, *Firmicutes*, *Lactobacilli* and *Streptococci* are lower compared to healthy controls. Active CD patients have an increased of gram-negative bacteria like *Bacteriodes*, *Bacterioidetesfragilis, Prevotella*, *E. coli*, *Proteobacteria*, *Haemophilus*, *Serratia*, *Klesbisella* [11,12]. Patients with active CD showed a strong presence of *Proteobacteria* phylum and *Neisseria flavescence*, *Firmicutes* and *Actinobacteria* are less abundant [13]. In the gut microbiota of CD patients, the presence of pathogenic bacteria like *Clostridium perfringens* and *C. difficile* may be a consequence of the *Bifidiobacteria* reduction and apparently seem to promote the risk of developing celiac disease in patient at risk [14]. Moreover, a reduction of *Bifidobacterium* and *Lactobacillus* population is present in these patients, that may be protective against enteric infections like infection due to *C. difficile*. Patients with high risk of CD showed a decreased of *Bacteriodes*, *Prevoltella* and *Bifobacteria*.

3.2. Changes in the GFD-CD Microbiota Respect to GCD-CD

CD patients at GFD for at least 2 years showed a faecal microbiota composition similar to healthy controls characterized by a reduced diversity of *Lactobacillus* and *Bifidobacterium* species indicating that GFD could normalize gut microbiota composition in CD [15]. Untreated celiac patients showed a lower concentrations of *Lactobacilli* and a significant higher concentration of *Clostridium* vs. healthy volunteers and CD relatives. The data are dependent on the time of the GFD suggesting that a prolonged GFD could modify SCFA proteolytic patterns. Dysbiosis with its imbalance of commensal microbiota composition could influence the metabolism of gluten proteins in CD patients [16,17].

3.3. Changes of the Microbiota of Healthy Subjects at GFD

The GFD in the healthy subjects influence the gut microbiota reducing the *Bifidobacterium*, *Clostridium lituseburense e Faecali bacterium praunsnitzii* and increasing the *Enterobac-*

teriaceae and *Escherichia coli* [18]. One study showed that the bacterial profile remained relatively stable in healthy individuals on GFD but during the GFD period decreased *Veillonellaceae*, *Ruminococcusbromii* and *Roseburiafaecis*. Whereas, *Victivallaceae*, *Clostridiaceae*, ML615J-28, *Slackia* and *Coriobacteriaceae* increased were described [19]. Moreover, using a low gluten diet, it is possible to observe an increase in unclassified species of *Clostridiales* and *Lachnospiraceae*. While, *E. hallii* and *A. hadrus*, *Dorea* and *T. blautia*, two species of the *Lachnospiraceae* and four species of *Bifidobacterium* decreased [20]. The GFD influences the gut microbiomain non-CD subjects increasing *E. Coli*, ML615J-28, *Slackia*, *Victivallaceae*, *Enterobacteriaceae*, *Clostridiaceae, Coriobacteriaceae* and unclassified specie of *Clostridiales* and *Lachnospriraceae*, decreasing *C. lituseburense, Lactobacillus*, *F. prausnitzii*, *Bifidobacterium, Dorea, B. wexlerae, Lachnospriraceae, A. hadrus, E. hallii, Veillonellaceae, R. bromi, R. faecis.*

In conclusion, CD patients present a different microbiome respect to normal subjects in the acute phase of the disease, while eating gluten. GFD can modulate gut microbiota of CD patients respect to healthy subjects, and can induce some alterations in the microbiota in non-CD subjects at GFD. Most studies on gut microbiota in CD are descriptive, including different categories of patients on GFD, GCD, at risk both with, and without, symptoms. From these studies it is difficult to determine whether the onset of CD is due to the alteration of microbiota or whether this is secondary to the intestinal damage present in CD.

In this context, pre-probiotics could have the characteristics to be useful to prevent or even ameliorate dysbiosis in CD. Post-biotics have not been tested in CD yet, but from the in vitro data they seem to be able to prevent some gliadin and gliadin peptides effects.

4. In Vitro Assays to Study Pro-Pre and Post Biotic in CD

Many pro-, pre- and post-biotics have been tested in intestinal epithelial cells in culture for their ability to prevent gliadin effects. The most used cell line is CaCo-2 an intestinal epithelial cell line derived from a human colorectal adenocarcinoma. Although, these cells transformed, they have the ability to differentiate in vitro toward intestinal epithelial cells. Once differentiated they produce tight junctions and absorptive-secretive ability.

The read outs used to study pro-, pre- and post-biotics in vitro were various. Generally, integrity of the epithelial layer, inflammation and innate immunity were analyzed. Following is an analytical division of pro-pre-post-biotics used and the main effects analyzedin vitro. The main read outs were tested before and after gliadin or gliadin peptides treatment. Following is a list of the main pro, pre and post-biotic used on this cellular model.

Principal read-outs used to study probiotics effect on CaCo-2 cells before and after gliadin or gliadin peptides treatment.

a. Permeability (TEER)
b. Toxicity experiments
c. Analysis of pro-inflammatory markers
d. Intestinal organoids

4.1. Permeability of Intestinal Epithelial Cells

Celiac disease is characterized by enhanced intestinal paracellular permeability probably due to alterations of tight junction (TJ) proteins function and expression. Intestinal permeability in CD and abnormal handling of gluten peptides by epithelial cells might activate the local immune system excessively and induce the disease [21]. TEER, one of the most popular methods to study permeability in epithelial cells is a non-invasive technique that consist of a measurement in electrical resistance across a cellular monolayer. It is regarded as a very sensitive and reliable method for confirming the integrity and permeability of the monolayer. The CaCo-2 monolayer generates a TEER of 150–400 Ω/cm^2 any insult that alter the monolayer will lower it. For these reasons, the CaCo-2 cells are often used to study nutrients and drugs transport. They also allow to study the main routes of transport [22].

4.2. Toxicity and Analysis of Pro-Inflammatory Markers

Gliadin peptides with specific amino acid sequences have proven to trigger pro-inflammatory cell responses. These involve activation of the nuclear factor kappa-B (NF-kB) in small intestinal mucosa of celiac patients [23], and increased expression of proinflammatory cytokines related to the innate immune response, such as tumor necrosis factor a (TNF-a) [24] and interleukine (IL) 1b [25].

5. Probiotics

5.1. Bifidobacteria

Bifidobacteria are Gram-positive microorganisms with a high G + C DNA content. In 1899 they were isolated from the faeces of a breastfed infant. They are strictly anaerobic and occur in uniform Y- and V-shaped branched and bifurcated, spatulate or club-shaped forms; they are not motile and do not form spores. The branched appearance of *bifidobacteria* depends not only on the strain but also on the medium used for cultivation. The genus *Bifidobacterium* consists of 48 different taxa, of which 40 have been isolated in the gastrointestinal tract (GIT) of mammals, birds or insects, the remaining eight from sewage and fermented milk [26–28]. Acetic and lactic acids are produced by glucose fermentation in a ratio of 3:2 [27]. There are 32 species of *bifidobacteria* (Table 1) identified by fermentation assays, observations of cell morphology, and electrophoretic mobility of enzymes such as transaldolase (14 types) or 6-phosphogluconate dehydrogenase (19 types; 32) [29].

Table 1. *Bifidobacterium* Species.

B. adolescentis *	*B. dentium* *	*B. minimum*
B. angulatum *	*B. gallicum* *	*B. pseudocatenulatum* *
B. animalis	*B.gallinarum* *	*B. pseudolongumsubsp. Globosum*
B. asteroides	*B. globosum*	*B. pseudolongumsubsp. Pseudolongum*
B. bifidum	*B. indicum*	*B. pullorum*
B. boum	*B. infantis* *	*B. ruminantium*
B. breve *	*B. inopinatum*	*B. saeculare*
B. catenulatum *	*B. lactis*	*B. subtile*
B. choerinum	*B. longum* *	*B. suis*
B. coryneforme	*B. magnum*	*B. thermophilum*
B. cuniculi	*B. merycicum*	

* Detected in human faeces.

Laparra and Sanz Y. (2010) demonstrated on CaCo-2 cells, the effects of *Bifidobacterium* strains (*B. IATA-ES2*, *B. longum IATA-ES1*, and *B. animalis IATA-A2*) by analyzing the peptide sequences produced by gastrointestinal digestion of gliadins and comparing toxic and proinflammatory effects.

By RP-HPLC-ESI-MS/MS different patterns of gliadin peptides were detected in samples inoculated with *bifidobacteria* compared to those not inoculated. Most of the peptides generated in the samples inoculated with *bifidobacteria* showed lower molecular mass than those generated in the non-inoculated samples (2500 Da) during the in vitro digestion [30].

Gliadin peptides resulting from in vitro digestion, inoculated or not, with *B. animalis* and *B. bifidum* were cytotoxic to intestinal epithelial cells in vitro. Whereas, those inoculated with *B. longum* were not. Furthermore, the analysis of pro-inflammatory cytokines showed that NF-kB, IL-1β TNF-a ($p < 0.05$) were consistently reduced when digested gliadin was inoculated with all bifidobacterial strains compared with non-inoculated digested gliadin [30].

Olivares M. et al. (2012) demonstrated by MALDI-TOPF techniques that digested gliadin up-regulates proteins involved in actin rearrangement, inflammation and apoptosis in CaCo-2, while *B. longum* CECT 7347 was able to reduce them (21 vs. 9). Many of these proteins were also involved in calcium homeostasis, cell survival and function [31].

de Almeida N et al. (2020) evaluated the effect of *Bifidobacterium* species: *B. bifidum, B. longum, B.breve, B. animalis* alone, and also a consortium of *Bifidobacterium* on the digestion of intact gluten proteins (gliadins and glutenins) and the associated immunomodulatory responses elicited by the resulting peptides. The readout was activation of NF-kB p65 and expression of the cytokines TNF-α and IL-1β in CaCo-2 cell cultures exposed to the peptides [32]. Giorgi A et al. (2020) used a multi-strain probiotic preparation containing two strains of *lactobacilli* (*L. paracasei, L. plantarum*) and three strains of *bifidobacteria* (two different *B. breve and B. animalis*). They evaluated the ability of a probiotic mixture to hydrolyze gluten peptides after simulated gastrointestinal digestion of gliadin (PT-gliadin). Protein and peptide mixtures, untreated or proteolyzed with the probiotic preparation, were analyzed before, and after, each proteolytic step by different techniques (SDS-PAGE, reverse-phase HPLC, filtration on different molecular shear membranes). PT-gliadin, untreated or digested with probiotics was then used to assess oxidative stress, IL-6 cytokine production, and expression of tight junction proteins, such as occludin and zonulin, in CaCo-2 cells. PT-gliadin induced IL-6 production and modulation and redistribution of zonulin and occludin, while digestion with probiotic strains reversed these effects [33] (See Table 2).

Table 2. In vitro study using *Bifidobacterium* and *Lactobacillus*.

Reference	Composition, Strains	Meaning
Laparra J.M. and Sanz Y. 2010 [30]	*Bifidobacterium strains (B. bifidum IATA-ES2, B. longum IATA-ES1, and B. animalis IATA-A2*	*Bifidobacterial* strains can inhibit the gliadin-induced cytotoxic and pro-inflammatory responses in intestinal epithelial cells
Olivares M et al., 2012 [31]	*Bifidobacterium longum CECT 7347*	*B. longum CECT 7347* reduces the toxic and inflammatory effects of gliadin-derived peptides
De Almeida and Natália Ellen Castilho et al. (2020) [32]	*Bacterium species B. bifidum BB-G90, B. longum BLG301, B. breve BB-G95and B. animalis L-G101*	*Bifidobacterium* species can able to assist the proteolysis of intact gluten proteins, gliadins, and glutenins, to form different peptide patterns, with reduction of cytotoxicity and proinflammatory response in intestinal epithelial cells
Giorgi A and et al., 2020 [33]	*Lactobacillus paracasei 101/37 LMG P-17504, Lactobacillus plantarum 14 D CECT 4528, Bifidobacterium animalis subsp. lactis Bi1 LMG P-17502, Bifidobacterium breve Bbr8 LMG P-17501 and Bifidobacterium breve BL10 LMG P-17500*	The probiotic strains tested canable to reduce the toxicity of gliadin

5.2. Lactobacillus

Lactobacillus, (genus Lactobacillus), are gram-positive, non-motile, ana- and aerobic, non-sporigenic bacteria. They are widely distributed in feed, silage, manure, milk, and dairy products. Despite continuous taxonomic changes in recent years, 70 species are recognized, and among them, 19 are of major interest in probiotic research (Table 3). From glucose metabolism, *lactobacillaceae* are able to produce lactic acid as a by-product, but also acetic acid, ethanol, carbon dioxide and other secondary compounds. For this reason, they are able to reduce pH and are used for the production of sour milk, cheese and yogurt, and play an important role in the production of fermented vegetables (pickles and sauerkraut), beverages (wine and juices), sourdough bread and some sausages. According to the type of Lactobacillus considered we have a different quantity of lactic acid produced: *L. acidophilus, L. casei and L. plantarum*, homofermentants produce 85% of it, primary by-product. In contrast, other species, such as *L. brevis and L. fermentum*, heteroferment glucose metabolism. *Lactobacilli* are commensal inhabitants of the animal and human gastrointestinal tracts, as well as the human mouth and vagina. Low levels of lactobacilli are present consistent findings in the microbiomes of adults and children with active CD [34].

Table 3. *Lactobacillus* Species Detected in the Intestinal Tract and/or used in Probiotic Products.

L. acidophilus	*L.delbrueckiisubsp. Bulgaricus*	*L. plantarum*
L. agilis	*L. gallinarum*	*L. rhamnosus*
L. aviarius	*L. gasseri*	*L. reuteri*
L. amylovorus	*L. johnsonii*	*L. murinus*
L. brevis	*L. hamsteri*	*L. ruminis*
L. casei	*L. intestinalis*	*L. salivarius*
L. crispatus		

Orlando A. et al. (2014/2018) have demonstrated that the administration of gliadin to CaCo-2 cells caused a significant alteration of paracellular permeabilityas by the rapid decrease in transepithelial resistance with a concomitant zonulin release causing also an increase polyamine content. The co-administration of viable *Lactobacillus rhamnosus GG*, heat-killed *L.GG* (L.GG-HK) or its conditioned medium (*L.GG*-CM) preserves the intestinal epithelial barrier integrity. Viable *L.GG* and L.GG-HK, but not *L.GG*-CM, led to a significant reduction in the single and total polyamine levels showing that the presence of cellular polyamines is a pre-requisite for this probiotic to exert its capability in restoring paracellular permeability by affecting the expression of different TJ proteins [35,36].

Sarno M. et al. (2014) have studied the effect of probiotic *Lactobacillus paracasei (LP) CBA L74* on the entrance of two peptides of gliadin: P31–43 and P57–68, the two main undigested peptides involved in CD pathogenesis, in CaCo-2 cells. Treatment of CaCo-2 cells with gliadin peptides, P31–43 and P57–68, conjugated with the fluorochrome lissamine (P31–43-liss and P57–68-liss) allowed to follow their entrance into the cells [37–39]. Pretreatment with *LP CBA L74* reduced the fluorescence intensity of both P31–43- and P57–68-liss inside CaCo-2 cells in a statistically significant way. Interestingly, *LP CBA L74* was more efficient in reducing P31–43-liss than P57–68-liss FI in CaCo-2 cells in a statistically significant way. This study describes a novel effect of probiotics [40].

6. Prebiotics

Pro-biotics are living microorganisms that mostly colonize the gastrointestinal (GI) tract. While, pre-biotics are fibres that cannot be digested or absorbed by the body. These act as a nutrient for probiotics, especially Bifidobacterium, increasing their numbers. They are defined as "non-absorbable food components that beneficially stimulate one or more groups of gut-friendly microbes and thus have a positive effect on human health" [41]. They can be metabolized by microbes in the gastrointestinal tract.

They positively affect the host by selectively stimulating the growth and/or activity of a limited number of bacteria. Naturally present in foods, such as artichokes, garlic, onions, and others. It may be necessary to consume large amounts of these foods to have a "bifidogenic" effect. For this reason, it is easiest to take a pre-biotic supplement or a combination of pro-biotic and pre-biotic (symbiotic) supplements to achieve optimal levels. They are, in addition, resistant to hydrolysis by digestive enzymes and are not absorbed in the upper part of the gastrointestinal tract. Upon reaching the large intestine, the site of the microbiota, they stimulate the growth of certain microorganisms [41]. Following their intestinal fermentation, SCFAs such as acetate, propionate and butyrate are produced [42].

Some of the most common pre-biotics are inulin and oligofructose. Oligosaccharides are non-digestible short-chain polysaccharides with numbers between 2 and 20 (approximately) saccharide units; hydrolysis of polysaccharides is a process that can be implemented to obtain different commercial products from dietary fiber, starch. An important pre-biotic is inulin, composed of a mixture of fructan chains and chicory roots are its main source of extraction. In vitro they stimulate the selective growth of Bifidobacterium [42].

In vitro, the use of prebiotics has not been studied, neither in CaCo-2 cells or in cells derived from CD patients. Many reports in the literature have been done in preventing the development of irritable bowel syndrome (IBS). For example, Qian Chen et al. (2017) explored the possible mechanisms using a prebiotic blend (PB) composed of fructo-

oligosaccharide (FOS), galacto-oligosaccharide (GOS), inulin and anthocyanins in CaCo-2 cells after co-incubation with PB and *Salmonella typhimurium* and in post-infectious IBS models in C57BL/6 mice. The results showed that PB significantly decreased pro-inflammatory cytokines in both infected CaCo-2 cells and PI-IBS models [43].

7. Post-Biotics

Post-biotics have been used in in vitro experiments in CaCo-2 cells daily to study their ability to prevent gliadin and gliadin peptides effects on CaCo-2 cells.

Sarno M. et al. have showed that *LP CBA L74* post-biotic was able to reduce gliadin peptides entrance in CaCo-2 cells [40].

Using 16S rRNA analysis of duodenal and oropharyngeal samples from CD patients and control subjects (Ctr), previously identified a peculiar *Neisseria flavescens* strain in adults affected by CD [13,44]. This bacterial strain, isolated from the above samples, induced an immune-inflammatory response in human and murine dendritic cells, in CaCo-2 cells, and in ex vivo duodenal mucosal explants of Ctr subjects, thereby suggesting that it could play a role in CD [13].

Labruna G et al. (2019) have evaluated if metabolism and trafficking was altered in CD-*N. flavescens*-infected CaCo-2 cells and if any alteration could be mitigated by pretreating cells with *LP CBA L74* supernatant, despite the presence of P31–43. CD-*N. flavescens* colocalised more than control *N. flavescens* with early endocytic vesicles and more escaped autophagy thereby surviving longer in infected cells. P31–43 increased colocalisation of *N. flavescens* with early vesicles. Mitochondrial respiration was lower in CD-*N. flavescens*-infected cells versus not-treated CaCo-2 cells, whereas pretreatment with *LP CBA L74* reduced CD-*N. flavescens* viability and improved cell bioenergetics and trafficking. CD-*N. flavescens* induces metabolic imbalance in CaCo-2 cells [45].

Sarno M. et al. have observed that *LP CBA L74* was able to ferment several different cereals. Fermented oats, rice and wheat were used to test the effect of *LP CBA L74* on P31–43 entrance after fermentation. P31–43-liss entrance was reduced after treatment with fermented oat (FI reduction 56%), rice (FI reduction 35%) and wheat (FI reduction 24%). These data suggest that *LP CBA L74* effect on P31–43-liss entrance was still present after fermentation of different cereals and that could be linked to compounds produced during the fermentation process and not to alive bacteria [40].

Gallo M. et al. have investigated if pH control during fermentation process of rice with probiotic *LP CBA L74*, could improve the bacterial growth and the lactic acid production and also the ability to interfere P31–43 entrance in the CaCo-2 cells. During rice fermentation process pH control greatly improved the lactic acid production. Both rice fermented with and without pH control, were able to prevent P31–43-liss entrance even after they were washed away from the cells, triggering into the cells a sort of memory that could prevent the entrance of P31–43-liss for several hours. This information could be useful in the field of nutrition; it suggests that the effect of certain post-biotics could last also after they have left the intestine [46] (See Table 4).

Recently, Freire R et al. using intestinal organoids developed from duodenal biopsies from both non-celiac (NC) and celiac (CD) patients, they analyzed the role of microbiota-derived molecules in modulating the epithelium's response to gluten. Therefore, the authors selected three bacterial bioproducts: Butyrate, lactate, and PSA derived from *Bacteroides fragilis*.

The authors analyzed the expression of genes related to gut barrier function, found altered in CD. Butyrate significantly upregulated the expression of MUC5AC, MUC6, TFF1 and CLDN18, in CD organoids. PSA significantly increased the expression of CLDN18 only, while lactate did not alter the expression of the analyzed gut barrier function-associated genes. Similar regulation of genes was observed in NC organoids.

Table 4. In vitro study using *LP CBA L74* post-biotc.

Reference	Composition, Strains	Meaning
Sarno M et al., 2014 [40]	*Lactobacillus Paracasei CBA L74*	The postbiotic *L. paracasei-CBA L74* interferes with gliadin peptides entrance in epithelial cells
Labruna G et al. (2018) [45]	*Lactobacillus Paracasei CBA L74*	*N. flavescens* strain induces imbalance in the mitochondrial respiration of CaCo-2 epithelial cells and was able to altered the trafficking pathway. This metabolic alteration appears to be in part reversed by the post-biotic *L. paracasei-CBA L74*, irrespective of the presence of the P31-43 peptide
Gallo M et al., 2015 [46]	*Lactobacillus Paracasei CBA L74*	During rice fermentation process pH control greatly improved the lactic acid production and also the ability to interfere P31-43 entrance in the CaCo-2 cells.

Moreover, they have analyzed the effect of the bacterial bioproducts on cytokines released by the CD monolayers challenged with gliadin, all bioproducts exerted a global protective effect by reducing the pro-inflammatory cytokine secretion triggered by PTG. However, only lactate and butyrate significantly reduced the secretion of IL15 and IFNγ cytokines, respectively in CD [47].

8. Clinical Trials Using Pro- and Pre-Biotic in CD

8.1. Probiotics and Celiac Disease

The intestinal microbiota has a very close relationship with the individual; provides a barrier against pathogen colonization, synthesize vitamins and other beneficial compounds, and stimulate the immune system. Celiac disease has been associated with a condition of dysbiosis, but no study has ever shown that there is a characteristic "celiac intestinal microbiota" [48]. The intestinal microbiota plays a fundamental role in maintaining and improving intestinal health and the whole organism [49]. Dysbiosis is present in the celiac patient, but it is still not clear whether this is the cause or consequence of the disease, it must be said that this dysbiosis persists regardless of adherence to a GFD and is partly related to this particular diet. Indeed, GFD influences the composition of the intestinal microbiota due to a reduction in the intake of polysaccharides [50]. Probiotics could help restore celiac dysbiosis. In this review, we analyzed various intervention studies conducted in the last 10 years (2011–2021), which envisaged the use of pro-biotics as an intervention. In particular, the analyzed works can be divided into two broad categories: Intervention studies, which evaluated whether the introduction of a pro-biotic could improve some parameters of subjects with CD in association with a GFD, and; studies which evaluated whether the administration of pro-biotics could influence the clinical course of the disease both in patients with untreated CD and in potential subjects (See Table 5).

Table 5. Effect of probiotics in vivo studying on GFD CD.

Reference	Intervation	Read Out	Effects
Olivares M et al. British Journal of Nutrition 2014 [51]	*B. longum CECT 7347* for 3 months in in thirty-three children	Evaluate the effects of *B. longum CECT 7347* administration on immune and anthropometric parameters, and on intestinal microbiota composition.	Reductions of pro-inflammatory bacteria (*B. fragilis group*), activated T lymphocytes and inflammatory markers (TNF-α)

Table 5. *Cont.*

Reference	Intervation	Read Out	Effects
Klemenak, M et al. *Dig Dis Sci* 2015 [52]	*Bifidobacterium breve BR03 and B. breve B632* for 3 months in 49 children	Investigate the effect of two probiotic strains on serum production of anti-inflammatory cytokine interleukin 10 (IL-10) and pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α)	Low production of TNF-α
Quagliariello et al. Nutrients 2016 [53]	*B. breve BR03 (DSM 16604) and B. breve B632 (DSM 24706)* for 3 months in 40 children	Impact of the administration of two *Bifidobacterium breve* strains on the gut microbiota composition	An increase of *Actinobacteria* and a re-establishment of the physiological *Firmicutes/Bacteroidetes* ratio
Harnett J et al. Evidence-Based Complementary and Alternative Medicine 2016 [54]	*VSL*#3 for 12 weeks 45 patients	Effects of a probiotic supplement on the CD microbiota	No effects
Francavilla R et al. J ClinGastroenterol. 2019. [55]	Mixture of 5 strains of lactic acid bacteria and *bifidobacteria* * for six months in 109 patients	Evaluate the efficacy and safety of probiotic mixture in CD patients with IBS-type symptoms despite a strict GFD.	Improving the severity of IBS-type symptoms, modification of gut microbiota, by an increase of *bifidobacteria.*

* *Lactobacillus casei* LMG 101/37 P-17504, *L. plantarum* CECT 4528, *Bifidobacterium animalis* subsp. *lactis* Bi1 LMG P-17502, *B.breve* Bbr8 LMG P-17501, *B. breve* Bl10 LMG P-17500.

8.1.1. Administration of Pro-Biotics in Patients with CD at GFD with or without Gluten Challenge

(a) Effect of pro-biotics associated with a gluten-free diet

In a study by Olivares M et al. [51] *B. longum CECT 7347* was given for three months to children with CD in combination with a GFD diet. The intervention resulted in; an increase in the height percentile compared to placebo treatment; significant reductions in mature T lymphocytes (CD3þ), and; HLA-DRþ T lymphocytes and reductions in TNF-a compared to placebo treatment. Moreover, in the study by Klemenak M et al. [52], the daily administration for three months of *B. short BR03* and *B632* in association with the GFD diet in subjects with CD led to a significant decrease in serum TNFα levels compared to the placebo group. In the study by Quagliariello A et al. [53] the administration of a mixture of 2 strains, *B. short BR03* (DSM 16604) and *B. short B632* (DSM 24706) (1:1) for three months in subjects with CD in association with GFD induced the restoration of the physiological *Firmicutes/Bacteroidetes* relationship in the intestinal microbiota, and led to an increase in members of the *Actinobacteria* phylum but this increase was not significant. In a multicenter study, the probiotic formulation VSL # 3 was administered for 12 weeks in 45 subjects with CD who reported only partial improvement in symptoms despite adhering to a strict GFD [54]. While, in the study of Francavilla R et al. [55], the 6-week intervention of a mixture of 5 strains of lactic acid bacteria and bifidobacteria: *Lactobacillus casei* LMG 101/37 P-17504, *Lactobacillus plantarum* CECT 4528, *Bifi-dobacterium animalis subsp. lactis* Bi1 LMG P-17502, *Bifidobacterium short* Bbr8 LMG P-17501, *B. short* Bl10 LMG P-17500, in patients suffering from CD at GFD with symptoms, led to a decrease in pain perception and a modification of the intestinal microbiota, characterized by an increase in *bifidobacteria* which is still detectable 6 weeks after stopping probiotics.

(b) Effect of pro-biotics on the gluten challenge in CD

In the study by Edgardo Smecuol et al. [56] the administration, in subjects with CD, for 3 weeks and of *Bifidobacterium infantis natren* life in conjunction with the daily con-sumption of 12 g of gluten led to a significant improvement in gastrointestinal symptoms compared to the placebo group. It is conceivable that the observed beneficial effect may be related to

the modulation of innate immunity. In this regard, a second study was conducted in which the administration *of B. infantis natren* Life Start super strain in sub-jects with CD at GFD with challenge of 12 g of gluten resulted in reductions in α-defensin and in the number of Paneth cells in duodenal biopsy samples [57].

8.1.2. Effect of Pro-Biotics in High-Risk Individuals

Subjects with positive genetics and first degree familiarity for celiac disease are regarded as "at risk".

The European multicentre Prevent Celiac Disease project investigated the possible primary prevention of celiac disease [58]. What they wanted to test was whether the frequency of celiac disease could be reduced by exposing children at high risk of the disease to small amounts of gluten between 16 and 24 weeks of age, preferably while still breastfeeding. The intervention consisted of randomly administering participants 200 mg of gluten mixed with 1.8 g of lactose daily for 8 weeks. The treatment did not reduce the risk of disease. In second study 832 infants were randomly assigned to two groups: Group A: gluten introduction at 6 months of age; Group B: gluten introduction at 12 months of age. Delayed gluten introduction did not change the risk of celiac disease among at-risk children, but delayed gluten introduction was associated with a delayed onset of the disease [59]. The Environmental Determinants of Diabetes in the Young (TEDDY) study evaluated whether the intake of *L. reuteri* and *L. rhamnosus*, during the first year of life, could influence the development of CD. The treatment had no protective effect [60].

8.1.3. Effect of Pro-Biotics in Potential Subjects

Potential Celiac Disease (PC) is characterized by the detection of specific antibodies in the serum, the presence of compatible HLA, but in the absence of alterations of the intestinal mucosa (Type 0, 1 according to Marsh). Patients with PC may or may not have clinical symptoms [8].

In the intervention study by Hakansson A et al., administration of *L. plantarum* and *L. paracasei* for 6 months in potential subjects on a normal containing gluten diet had modulatory effects on the peripheral immune response; but it showed no effect in preventing the progression of the disease, which developed in the same proportion in both groups [61].

9. Pre-Biotics as Food Supplements in GFD

The gluten-free diet exposes celiacs to nutritional deficiencies. In fact, the consumption of some nutrients, in particular fiber, iron, calcium and folic acid, is lower than normal in patients who follow a gluten-free diet. One possible strategy for avoiding the nutritional deficiencies in CD patients is appropriate dietary supplementation. According to the most recent definition, proposed by the International Scientific Association of Pro-biotics and Pre-biotics in 2017, pre-biotics are substrates that are used by the intestinal flora as an energy source; these substrates have a beneficial effect on human health [62]. Prebiotics can have beneficial effects on the absorption of vitamins and minerals; some of them have been shown to improve mineral bioavailability and increase iron absorption in animal studies [63].

To date, all interventions with pre-biotics have been tested in celiac subjects at GFD with the aim of improving the subjects' nutritional status.

In a randomized clinical trial with a placebo conducted on 34 patients with celiac disease, diagnosed according to the criteria created by the European Society of Gastroenterology, Hepatology and Pediatric Nutrition (ESPGHAN criteria 2012) the administration of 10 g per day of Sinergy 1 (oligofructose-enriched inulin, ORAFTI, Tienen, Belgium) resulted in a significant reduction of hepcidin (regulator of iron metabolism in the intervention group). No differences were found in the morphological and biochemical parameters of the blood (for details see Table 6) [64]. Natalia Drabińska et al. observed that patients with GFD may counteract the reduction in *Bifidobacterium* count in CD children after the administration of Synergy 1. The addition of Synergy 1 modified the faecal SCFA profile,

and in particular, increased the concentration of acetates. In a second intervention study, the levels of 25-hydroxyvitamin D [25 (OH) D], parathyroid hormone, vitamins E and A, calcium, phosphate, magnesium, total protein and albumin were evaluated after administration of 10 g a day of Sinergy 1 for three months. The Synergy 1 leds to an increase in 25 (OH) D and vitamin E, but there was no difference in the levels of parathyroid hormone, calcium, phosphate and vitamin A [65,66]. The circulating amino acid (AAs) concentrations are indicators of dietary protein intake and metabolic status. In CD, the AA imbalance is frequently observed. In two other papers, the integration of Sinergy 1 for three months led to an increase in urinary excretion of AA with a simultaneous increase in the content of circulating AAs, which could be attributed to higher absorption and/or intensified metabolism of AAs, as well as further healing of the intestinal mucosa [67]. Changes in Glu concentration suggest that oligofructose-enriched inulin could improve the intestinal condition and permeability, leading to benefits in bone metabolism, increasing bone formation rates and reducing bone resorption rates [68].

Table 6. Effect of prebiotics in vivo study.

Reference	Read Out	Effects
Feruś, K et al. Nutrients 2018 [63]	Evalutate the effect of oligofructase-enriched inulin (Synergy 1) on iron homeostasis in CD children GFD	Reduction in serum hepcidin concentrations, no change in blood morphological and biochemical parameters (including ferritin, hemoglobin and C-reactive protein (CRP)
Drabińska, N et al. Nutrients 2018 [65]	Evaluate the effect of prolonged oligofructose-enriched inulin (Synergy 1) administration on the characteristics and metabolism of intestinal microbiota in CD children GFD	Increase *Bifidobacterium* and modified the faecal SCFA profile, increasing the concentration of acetate
Drabińska, N Amino Acids 2018 [67]/Nutrients 2018 [66]	Analyze the effect of the intervention on plasma and urinry concentrations of AA	Synergy 1 could improve the intestinal condition and permeability
Drabińska, N et al. Nutrients 2018 [67]	Determine the variations of 25-hydroxyvitamin D[25(OH)D], parathyroidhormons, vitamines E and A, calcium, phosphate, magnesium, total protein and albumin	Improved vitamin D and vitamin E status in children and adolescents with CD
Drabińska, N et al. Bone 2019 [68]	Effect of oligofructase-enriched inulin (Synergy 1) on bone turnover markers and immune response in children in CD children GFD	Improved bone metabolism increased bone formation rates and decreased bone resorption process rates. The intervention did not lead to immunological response changes

Type of study: Randomized double blind. Sample size: 34 children. Follow up: 3 months. Inclusion criteria: ESPGHAN 2012. Intervation: 10 g of oligofructose-enriched inulin (Synergy 1) or a placebo (maltodextrin).

10. Conclusions

The few reports of in vivo trials with pro-biotics in CD indicate that they are not able to prevent the disease. Pro-biotics only seem to be effective in alleviating symptoms. These conclusions are in line with other reports on Crohn's Disease and Ulcerative Colitis. Pre-biotics alone do not seem to be very active in vivo, whereas post-biotics have never been used in vivo.

In vitro post-biotics in intestinal epithelial cells seem to be very effective in preventing gliadin and gliadin peptides effects. Post-biotics seem to be effective in many different pathways that ultimately point to the prevention of the inflammation induced by gluten. The use of in vitro patient-derived organoids to model CD pathogenesis could be a novel tool to further study CD treatment and prevention

It is not clear what aspect of the post-biotic induces this activity, although some reports point to small protein/s produced by the bacteria. Taken all together, these data point to a

possible application of post-biotics in the prevention of inflammation in CD patients at risk of the diseases. Only in vivo trials will be able to confirm this application.

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