Probiotics supplements reduce ER Stress and gut inflammation associated with gliadin intake in Celiac Disease mouse model

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Abstract: Celiac disease (CD) is a permanent intolerance to dietary protein, gluten, from wheat rye and barley. It occurs in about 1% worldwide population, in genetically predisposed individuals bearing human leukocyte antigen (HLA) DQ2/DQ8. Gut epithelial cell stress and the innate immune activation are responsible for the breaking oral tolerance to gliadin, the gluten component. To date, the only treatment available for CD is a long-term gluten-free diet. Several evidences show that an altered composition of the intestinal microbiota (dysbiosis) could play a key role in the pathogenesis of CD, through the modulation of intestinal permeability and the regulation of the immune system. Here we show that gliadin induces a chronic ER stress condition in the small intestine of a CD mouse model and that the co-administration of probiotics efficiently attenuates both UPR and gut inflammation. Moreover, the composition of probiotics formulations might differ in their activity at molecular level, especially toward the three axes of the UPR. Therefore, rebalancing the gut microbiota composition by probiotics administration might represent a new strategy to treat CD affected patients.

Keywords: CD; UPR; TG2; CFTR; probiotics

1. Introduction

Celiac Disease (CD) is a chronic autoimmune enteropathy caused by exposure to gluten protein from wheat, rye, and barley. CD occurs in about 1% of worldwide population in a genetically predisposed individual carrying Human Leukocyte Antigen (HLA) DQ2/DQ8 [1]. In CD patients, gliadin, a gluten component, binds the chemokine CXC motif receptor 3 (CXCR3), thus promoting the release of Zonulin, resulting in tight junctions disassembling, with gliadin crossing the epithelial barrier. Next, deamidated gliadin, through tissue transglutaminase 2 (TG2) activity, can bind the HLA-DQ2/DQ8 molecules on APCs [2,3] and then presented to CD4+T cells, resulting in their activation and migration to the small intestinal lamina propria [4]. Once in the lamina propria, activated CD4+T cells proliferate and start to produce proinflammatory cytokines, such as IFNγ, and metalloproteases, and stimulate the production of growth factors by stromal cells, which induces crypt hyperplasia and villous atrophy, due to intestinal epithelial cells death induced by intraepithelial lymphocytes (IELs) [5]. Recent studies indicated a new actor playing a key role in the pathogenesis of CD, the cystic fibrosis transmembrane conductance regulator (CFTR) [6], linking CD to Cystic Fibrosis (CF). CF is the most common genetic lethal disease in Caucasian population, caused by loss-of-function mutations of the cftr gene.
gene. Although CFTR was originally identified as a cAMP-activated transmembrane anion channel mediating the transport of Cl-/HCO3- across the epithelia, it is now also recognized as a hub protein regulating and orchestrating a complex protein network in epithelial cells. Loss of function mutations of CFTR cause an increased reactive oxygen species (ROS) production, activation of TG2, inhibition of autophagy and a defective bacterial killing [7–9]. Moreover, active TG2 leads to NF-κB activation then causing an increased level of pro-inflammatory cytokines such as IL-15, IL-17A and IL-21, the main cytokines involved in CD pathogenesis. Importantly, gliadin binds to the NBD1 domain of CFTR thus inhibits its gating functions, impairs autophagy and proteostasis [6,10]. To close this vicious circle, inhibited CFTR sustains, in turn, the activity of TG2 which enhances the amount of deamidated gliadin, finally raising its antigenicity [10]. Importantly, the above-mentioned activation of TG2 seems to rely on gliadin-stimulated intracellular calcium mobilization from the Endoplasmic Reticulum, a condition potentially resulting in imbalanced ER homeostasis known as ER Stress [11], as evidenced in vitro by Caputo and colleagues [12]. Importantly, perturbed ER homeostasis seems to be a feature of inflammatory intestinal disorders such as IBD [13], although its role is still under investigation.

Actually, the only effective treatment for CD is a long-life gluten free diet. However, many patients have many difficulties to adhere to this restriction diet throughout their life. However, new therapeutic approaches have been suggested and include Zonulin receptor inhibitors, engineered gluten-free grain, TG2 inhibitors, and probiotics addition to the diet [14–16]. The latter intervention is focused on buffering the gut microbiota dysregulation, which has been described to influence the CD pathogenesis through the modulation of intestinal permeability, regulating the immune system, and modulating the digestion of gluten generating toxic and tolerogenic peptide [17]. Indeed, gut microbiota dysbiosis has been associated to the development and progression of several chronic gut diseases such as IBD [18], Colitis [19] and CD [20]. Interestingly, dysbiosis often persists unexpectedly in some CD patients despite the gluten-free diet, with this condition potentially and surprisingly being induced by this restricted diet.

In the present study, we tested the hypothesis by which restoring the gut microbiota equilibrium by means of probiotics supplements might alleviate the CD associated intestinal dysfunctions due to gluten intake, by using our previously established mouse model of CD [6].

2. Materials and Methods

2.1 Mice and treatments

Balb/c mice were obtained from Charles River (Calco). 8 weeks old of three-generation gluten free mice (Mucedola), for a total of 36 mice, were randomly divided into 6 groups (G1-G6), composed of 6 mice/group. The G1 was challenged with a gluten free diet for all along with the time of the experiment; the G2-G4 were challenged via oral gavage with gliadin (Sigma; 5mg/daily for 1 week, then 5 mg/daily thrice a week for 3 weeks) for 4 weeks [6,21]. At the end of the fourth week, mice from G2-6 were challenged with gliadin alone (G2) or in combination with P1 (G3) or P2 (G4), or with P1 (G5) or P2 (G6) alone, every day via oral gavage, for another two weeks (P1: 5,7x10^5±2.0x10^5/day; P2: 8,5x10^5±2.0x10^5/day; equivalent doses in mice, calculated on the basis of those recommended for humans). A schematic representation of treatments is reported in Supplementary Figure S1. At the end of the last daily treatment (7 weeks), all mice were sacrificed and the intestine and the blood were collected and used for the analysis described below.

All procedures were approved by the local Ethics Committee for Animal Welfare (IACUC No 849) and conformed to the European Community regulations for animal use in research (2010/63 UE).

2.2 Cell lines and treatments
Human colon adenocarcinoma-derived Caco-2 cells were obtained from ATCC. Cells were maintained in T25 flasks in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS) 2mM glutamine (Sigma) and 1% penicillin/streptomycin (Sigma). 3x105 cells/well were seeded in a six well plate and treated or untreated 3 or 9h (with or without refresh of the treatment every 3h, as indicated) with pepsin-trypsin-gliadin (PT gliadin; 1mg/ml) with or without 1x109 bacterial cells of probiotic formulation 1 (P1) or 2 (P2). PT was prepared as previously described [22] with minor modifications. Briefly, 50g of gliadin (Sigma) was dissolved in 500ml 0.2N HCl for 2h at 37°C with 1g of pepsin (2,5 units/mg protein; Sigma). The resultant peptic digest was further digested by addition of 1g trypsin (Sigma) after pH adjusted to 7.4 using 2M NaOH. The solution was stirred vigorously at 37°C for 4h, boiled to inactivate enzyme for 30 minutes, lyophilized and then stored at -20°C until used. PT gliadin was freshly resuspended in a sterile phosphate buffered saline (PBS). A schematic representation of the experimental procedure is reported in Figure 5A.

2.3 Probiotics formulations

Probiotics were supplied by PROBIOTICAL Research Srl, in a lyophilized format. The P1 formulation contains two strains of Bifidobacterium breve, the B632 and BR03 (4x109 bacteria/g), while the P2 formulation contains the Lactobacillus plantarum LP14, L. casei subsp. paracasei LPC09 and the Lactobacillus rhamnosus LR04 (3x109 bacteria/g). P1 or P2 were resuspended in PBS and administrated as described. The probiotic solution prepared is plated before every gavage to get an accurate count of CFU administered.

2.4 Intestinal permeability assay

The fluorescein isothiocyanate conjugated dextran (FITC-Dextran 4000; Sigma) was used to perform the intestinal permeability assay by using 4 animals/group of Balb/c mice treated as described in the previous section. Briefly, FITC-Dextran was oral gavaged to the mice at a concentration of 44mg/100g body weight, 4 hours previous the euthanasia. At the end of treatments, mice were anesthetized and blood was collected by cardiocentesis, heparinized, centrifuged 10 min at 12000xg, and plasma was protected. Next, each plasma was diluted with an equal volume of phosphate-buffered saline (PBS, pH 7.4), and a standard curve was obtained though serially diluted FITC-Dextran stock solution (0; 125; 250; 500; 1000; 2000; 4000; 8000 ng/ml). 100µl of each diluted plasma (in quadruplicate) was transferred to a 96-well microplate, fluorescence (485nm ex; 528nm em) was evaluated by using a SPARK Multimode Microplate Reader (TECAN), and the FITC-Dextran concentration was obtained by using the standard curve interpolation.

2.5 Western blotting analysis

The whole small intestine lysates were obtained by using the Cell Lytic buffer (Sigma) supplemented with a protease inhibitors cocktail (Sigma) plus phosphatases inhibitors (Na3VO4 1 mM; NaF 10 mM), and resolved by electrophoresis through SDS-PAGE, and electroblotted onto nitrocellulose (Protran, Sigma) membranes. Membranes were incubated with indicated primary antibodies in 5% non-fat dry milk (Bio-Rad) in PBS plus 0.1% Tween20 overnight at 4°C. Primary antibodies were: anti-CFTR (clone M3A7 Abcam ab4067) 1:500, anti-TG2 (NeoMarkers) 1:750, and anti-βActin (Cell Signaling) 1:2000. Detection was achieved using horseradish peroxidase-conjugate secondary antibody (1:5000; Jackson ImmunoResearch; Cambridge, UK) and visualized with ECL plus (Amersham Biosciences; Amersham). Images were acquired by using a ChemiDoc™ Touch Imaging System (Bio-Rad) and analyzed by Image Lab software (Bio-Rad), as previously described (Giglio et al., Genes & Immunity 2018).

2.6 ELISA
IL-15, IL-17A, and INFα were measured in small intestine lysates by using the Mouse IL-15 DuoSet ELISA, the Mouse IL-17 Quantikine ELISA Kit, or the Mouse IFNα Quantikine ELISA Kit (Bio-Techne), as recommended by the supplier. ODs were analyzed by a SPARK Multimode Microplate Reader (TECAN). Values were normalized to total protein concentration evaluated by Bradford analysis, as previously reported (Villella et al., 2019).

2.7 Hematoxylin/Eosin staining
After surgical removal of the small intestine, samples were fixed in formalin buffer at room temperature, dehydrated and embedded in paraffin. 8μm thick sections were collected by using a microtome (Leica). All sections were mounted on slides, stained with hematoxylin and eosin (Bio Optica) and images were acquired by using a Nikon Eclipse Ci Microscope, a Plan APO 10X Objective, and the NIS-Elements Software (Nikon).

2.8 Quantitative RT-PCR
Trizol reagent (Invitrogen) was used to isolate total RNA, as indicated by the supplier. The AMV Reverse Transcriptase kit (Promega) was used to generate cDNA following the manufacturer’s recommendations. Quantitative PCR reactions were performed by using the CFX96 thermocycler (Bio-Rad). Supplementary Table 1 shows the primers sequence for all amplicons, designed by using the online IDT PrimerQuest Tool software (IDT, Integrated DNA Technologies Inc., USA; https://eu.idtdna.com/Primerquest/Home/Index). Results were normalized by using mouse GAPDH or human L34 as internal control (Antunes et al., Cells 2020).

2.9 Statistical analysis
All experiments were performed at least in triplicate and statistical analysis was performed using GraphPad Prism 6. The Student’s t test was used to determine statistical significance. A p-value of equal to or less than 0.05 was considered significant. In each table: **** p<0.0001; *** p<0.001; ** p<0.01; * p<0.05.

3. Results
3.1 Probiotics administration inhibits gliadin-mediated TG2 upregulation but does not restore CFTR physiological expression.
CFTR and TG2 are two key players in CD, since CFTR activity is inhibited by gluten derived peptides, resulting in protein destabilization and subsequent degradation (Villella 2019). CFTR impairment also results in TG2 expression upregulation and activation which promotes the TG2-mediated gliadin peptides deamidation which, in turn, causes an increased binding affinity of deaminated peptides to the disease-predisposing human leukocyte antigen (HLA) DQ2 and DQ8 molecules, thus enabling a strong immune response contributing to the pathogenesis of celiac disease.

Therefore, we evaluated both CFTR and TG2 mRNA and protein levels in the small intestine of Balb/c gluten sensitive mice exposed to gliadin for four weeks and for two more weeks to gliadin in presence or absence of P1 or P2 probiotics formulations. Data reported in figure 1 show that gliadin exposure efficiently downregulated the expression of CFTR (A) and consistently elevated the expression of TG2 (B), at both mRNA and protein levels. These results clearly indicate that gliadin exposure was able to ignite a typical gut dysfunction observed in CD patients. Importantly, the concomitant administration of P1 or P2 efficiently inhibited the gliadin-induced TG2 upregulation, at both mRNA (Fig. 1B, right panel) and protein (Fig. 1B, left panel) levels, suggesting the ability of these probiotics formulations to potentially reduce the damaging effect exerted by gliadin peptides.
Moreover, our data also show that the two probiotic formulations were able to restore at least in part the physiological protein levels of CFTR (Fig. 1A, right panel), while no major effects were observed at mRNA levels (Fig. 1A, left panel). Further studies are therefore required to better investigate this aspect.

Collectively, these data indicate that the bacteria from the two formulations do not prevent the formation of the active gliadin peptides generated by digestion, nor do they interfere directly (via cell-cell contact) or indirectly (via metabolites) with the effects of the latter molecules with the CFTR present in the cell membrane of intestinal epithelial cells, but nevertheless exert their beneficial activity downstream of this event, as confirmed by restored physiological TG2 expression.

3.2 Dysregulated intestinal permeability due to gliadin exposure was restored by probiotics administration.

Altered intestinal permeability is one key features of CD pathogenesis. In fact, although the ability of gliadin peptides to cross the intestinal epithelial barrier is prevented by the presence of an efficient Zonulae occcludent established among epithelial cells under physiological conditions, through means of thigh junctions (TJs), in genetically susceptible individuals, however, intestinal cells trigger TJs disassembly. Indeed, during the acute phase of CD, it has been reported that zonulin, a protein involved in the regulation of intestinal permeability, is upregulated. When zonulin binds to its surface receptors leads to an opening of the tight junctions, resulting in an increase in intestinal permeability [23].

Therefore, we evaluated the intestinal permeability in vivo, in mice exposed 6 weeks to gliadin, by using FITC-Dextran administrated as a single dose (through gavage; 44mg/100g body weight) 4h prior animal sacrifice [6,21] Fluorescence (FITC) measured in the plasma of mice and reported in figure 2A shows a considerable increased permeability.
in mice exposed to gliadin, compared to untreated control mice. These data are in line with the increased upregulation of both claudin 2 and 15 and downregulation of occludin (three TJ components), observed in mice exposed to gliadin compared to controls, and previously associated to impaired intestinal permeability (Fig. 2B) [24–26]. Importantly, the co-administration of P1 or P2 consistently inhibited the intestinal permeability impairment mediated by the gliadin active peptides, as shown in figure 2A. Moreover, these results were confirmed by the restored claudin 2 and 15 physiological expression in mice exposed to gliadin in presence of P1 or P2 (Fig. 2B upper right and bottom left panels, respectively).

Figure 2. Gliadin-mediated intestinal permeability dysregulation is restored by probiotics administration. Plasma concentration of FITC fluorescence was measured 4h after mouse gavage of a single dose of FITC-Dextran (A). Quantification of plasma concentration from n=2 mice per group. Mean ± SD of duplicate sample. (B)

Claudin 2 (upper panel), Claudin 15 (bottom left panel) and Occludin (bottom right panel) expression levels were evaluated in small intestine of Balb/c mice fed with three generation gluten free diet challenged with gliadin (Glia), in presence or absence of P1 or P2, by qRT-PCR. Mean ± SD of triplicate sample; **** p<0.0001; *** p<0.001; ** p<0.01.

3.3 Gliadin-mediated small intestinal inflammation was buffered by probiotics administration.

CD is a T-cell mediated disease, in which, gliadin-derived peptides activate T lymphocytes infiltrating the lamina propria, resulting in the production and release of pro-inflammatory cytokines. Indeed, we observed the upregulation of key pro-inflammatory cytokines, IFNγ – IL-15 – IL-17A [6,21], in small intestine lysates of animals exposed 6 weeks to gliadin, compared to untreated controls (Fig. 3A), confirming the gliadin-induced small intestine pro-inflammatory condition. Importantly, in line with data reported above, the presence of P1 or P2 consistently inhibited the upregulation of the pro-inflammatory genes and cytokine release (Fig. 3A and B, respectively). However, although both probiotics formulations completely inhibited the gliadin-mediated upregulation of IFNγ, which is secreted by Th1 cells and promoting the release of degrading enzyme involved
in the damage of intestinal mucosa, such as metalloprotease (Ferretti et al., 2012), a different effect was observed against the other two cytokines.

In particular, while P2 was more efficient (compared to P1) in inhibiting the gliadin-mediated IL-15 up-regulation and secretion, previously reported to be upregulated in CD and leading to an increase in intraepithelial lymphocytes [27] (Fig. 3A and B, bottom panels, compare the two rightmost conditions, P1 and P2), the P1 was the most efficient in reducing the gliadin-mediated IL-17A up-regulation, which is involved in the pathogenic effect attributed to Th1 cells in CD, compared to P2) [28] (Fig. 3A, middle panel, compare the two rightmost conditions, P1 and P2).

Collectively, our data confirm a prominent small intestinal inflammation induced by long term gliadin exposure compatible with the human CD condition, and importantly show that probiotics administration consistently mitigates the toxic effects of the active gliadin peptides, although with a different extent, depending on the specific probiotic composition.

Importantly, the ability of probiotics in mitigating the pro-inflammatory and tissue damage activity of gliadin exposure were also evidenced by morphological analysis of small intestine section, as reported in figure 3C. In fact, H/E staining of tissue from gliadin exposed mice show a clear change in the morphology of villi (atrophy) and immune cells infiltration, compared to matched control. Combined gliadin and P1 or P2 treatment completely reverted the gliadin-mediated tissue damage (Fig.3C).

3.4 ER stress was promptly induced by gliadin and efficiently inhibited by probiotics administration.
The endoplasmic reticulum (ER) is the site of synthesis and folding of lysosomal, membrane and secretory proteins, which, collectively, represent a large fraction of the total protein output of a mammalian cell. The homeostasis of this compartment and, therefore, its function is finely regulated by calcium concentration, redox potential and availability of chaperonins and co-chaperonins. Extracellular or intracellular insults compromising the homeostasis of this organelle results in an impaired function termed ER Stress consisting in a luminal accumulation of misfolded proteins which, in turn, activates the so-called Unfolded Protein Response (UPR) [11]. The UPR function is primarily a pro-survival response aimed to restore the physiological functions of this compartment, through the activation of a finely regulated genetic program. However, acute or unsustainable stress will result in the activation of a UPR-mediated pro-apoptotic program [29,30]. Therefore, due to the key role played by ER and UPR in cell functions and stress management, it is not surprising that ER Stress (and UPR) has been implicated in the pathogenesis of many diseases and in particular in inflammatory disease, potentially contributing substantially to disease onset and progression [31]. In this context, Caputo and colleagues indicated the potential induction of ER Stress in vitro, in Caco-2 cells exposed to gliadin [12]. To validate this hypothesis in vivo, we evaluated the activation of UPR in our mouse model of CD, in animal exposed 6 weeks to gliadin, compared to unexposed controls. Data reported in figure 4 clearly show a consistent upregulation of the three main ER stress markers such as ATF4, ATF6 and XBP1 (Fig.4).

Figure 4. ER stress induced by gliadin exposure was buffered by probiotics. ATF4 (A), ATF6 (B) and XBP1s (C) expression levels were evaluated in the small intestine of Balb/c fed three generation gluten free diet treated (Glia) on untreated (CTRL) with gliadin, in presence or absence of P1 or P2. Mean ±SD of triplicate sample; **** p<0.0001; *** p<0.001; ** p<0.01; ns = not significant.

Interestingly, ATF6 and XBP1 seem to be the most upregulated, compared to ATF4, and collectively this is compatible with a chronic stress condition in which the UPR-related signaling pathways particularly involved in the degradation of misfolded proteins (ERAD) and gene expression regulation of chaperonins/co-chaperonins, regulated by XBP1 and ATF6, are highly active, compared to that particularly involved in the regulation of protein synthesis (PERK/eIF2α/ATF4 axis).

Importantly, the exposure of gliadin-treated mice to P1 or P2 completely abrogated the gliadin-mEDIATE upregulation of both ATF4 and XBP1 (Fig.4A&C, respectively). Interestingly, while the expression of ATF6 was completely inhibited by P1, in the same experimental conditions, P2 failed since the levels of this factor remained elevated (Fig. 4B, compare the two rightmost conditions). Further studies are required to define the role of elevated ATF6 in presence of gliadin and P2.

Therefore, our data indicate that ER stress is involved in the pathogenesis of CD in vivo and that probiotics might be used to efficiently restore the homeostasis of this compartment, potentially buffering the gut inflammation in CD patients.

### 3.5 Probiotics were able to efficiently inhibit the gliadin-mediated ER stress but did not restore physiological levels of CFTR and TG2, in vitro.

Finally, we tested the ability of the two probiotics formulation to restore the physiological ER homeostasis also in vitro, by using Caco-2 cells as a model. To this aim, we
exposed Caco-2 cells to pepsin-trypsin-gliadin digested peptides (PT) for 3 and for more 6 hours in presence or absence of P1 or P2, while untreated cells were used as control. Importantly, the medium containing PT±P1 or P2 was replenished every 3h, as reported in Figure 5A, since after 3h of treatment it seems that PT loses its toxic activity (Supplementary Figure S2). Our data confirmed that PT efficiently downregulated the expression of CFTR as soon as 3h, and its low expression level was stable at 9h post treatment (Fig.5B). In parallel, we observed a prompt upregulation of TG2 (at 3h), still evident after 9h of PT treatment (Fig.5C). In the same experimental conditions, we also confirmed the induction of UPR upon PT treatment, at both 3 and 9h (Fig.5D-F). Moreover, the presence of P1 or P2 failed in restoring the physiological expression levels of CFTR, at least at mRNA level, confirming our in vivo data (Fig.1A). In parallel, in contrast to in vivo data, both P1 and P2 were not able to inhibit the PT-mediated enhanced expression of TG (compare Figure 1B and Figure 5C), potentially indicating a microenvironment involvement, in vivo.

Of note, both probiotics formulations efficiently and completely abrogated the PT-induced ER stress, as evidenced by the complete inhibition of both ATF4 and XBP1 up-regulation (Fig.5D&F). Importantly, although P1 also completely abrogated the PT-stimulated upregulation of ATF6, the expression levels of this factor were lower but still high in presence of P2 (Fig.5E, compare the two rightmost histograms), confirming our in vivo data indicating a different activity of P1 and P2 on the gliadin-mediated altered expression of ATF6 (Fig.4 middle panel).

Figure 5. Impact of probiotics on PT-mediated ER stress induction in vitro. Caco-2 cells were untreated (CTRL) or treated with PT-gliadin for 3 (PT 3h) or 9 (PT 9h) hours in presence or absence of P1 or P2 as schematically reported in panel A, and CFTR (B), TG2 (C), ATF4 (D), ATF6 (E) and XBP1s (F) expression levels were evaluated by qRT-PCR. Mean ±SD of triplicate sample. Mean ±SD of triplicate sample; **** p<0.0001; *** p<0.001; ** p<0.01; * p<0.05; ns = not significant.

4. Discussion

Celiac Disease represents a global health problem, with a prevalence around 0.5%–1% in the general population, and approximately 1% of the population in the Western world [32,33]. CD is an autoimmune chronic disorder producing intestinal damages induced by gluten and gluten-related proteins, and in which genetic susceptibility plays a key role. In fact, the consumption of gluten and/or gluten-related proteins by subjects with genetic predisposition represents the main environmental risk factor for CD. A major role in the ignition of immune response toward gliadin peptides produced by partial enzymatic digestion in the intestine is played by tissue transglutaminase (TG2). This enzyme is able to deaminate the glutamine rich gliadin-derived, resulting in an enhanced affinity...
to HLA-DQ2 or DQ8, finally resulting in downstream activation and CD4+ T cell-mediated response [34]. Such response initiates a cascade resulting in intestinal inflammation, villi atrophy, and enteropathy, that can produce extended damage in the mucosa of the small intestine [35].

Although several clinical interventions have been proposed, the only worldwide-accepted treatment for CD is a strict, lifelong, gluten-free diet (GFD). Although the majority of CD patients fully respond to GFD and have a normal life expectancy, elderly people, diagnostic delay, and poor adherence to GFD represent risk factors to develop disease complications such as refractory celiac disease (RCD), enteropathy-associated T-cell lymphoma (EATL), and small bowel carcinoma (SBC) [36–38].

The GI tract is also a complex ecosystem in which the microbiota has been recently described as an ‘extra organ’ of the body. It is mainly constituted of bacteria, as well as archaea, viruses, protozoa and fungi [39]. Adult human gastrointestinal tract harbors about trillions of bacteria, including at least several hundred species and more than 6000 strains. However, this is not an isolated ecosystem but, on the contrary, it is intensely and actively connected with the host, via a bidirectional intense communication. Indeed, it plays key roles in GI functions such as: microbes facilitate the digestion and transformation of indigestible polysaccharides, provide vitamins, participate to the shaping of the intestinal epithelium, are involved in host immune defense against pathogens in the intestinal lumen, and contribute to the maintenance of intestinal homeostasis [39]. Although the community of the GI microbiota does not undergo significant fluctuations throughout adult life, antibiotic exposure, infections, lifestyle, and diet might profoundly affect it. Therefore, it is not surprising that altered microbiota homeostasis (dysbiosis) has been linked to the onset/progression of diseases characterized by inflammation of the GI tract, such as Crohn’s Disease, Ulcerative Colitis [40] and CD [41]. Importantly, the strong impact of microbiota on host health is not restricted to those pathologies, but has also been evidenced in other human diseases ranging from cardiovascular, neurologic, respiratory and metabolic illnesses to cancer [42]. However, the key question that arises with a not yet convincing answer is: does dysbiosis precede or is it a consequence of disease?

Nonetheless, buffering the gut dysbiosis seems to offer a new treatment opportunity to mitigate, delay, or inhibit the progression of several human disorders. Indeed, in this context, a diet supplementation with probiotics and prebiotics have been explored as a strategy to modulate the gut microbiome to an anti-inflammatory state. In line with these hypotheses, in the present study, we demonstrated that probiotics administration efficiently reduces the hallmarks of intestinal inflammation stimulated by gliadin, in a mouse model of CD.

In conclusion, our data clearly indicate that ER stress is involved in the pathogenesis of CD in vivo, with the involvement of the whole signaling pathway, as demonstrated by the concomitant activation of the three main axes such as the PERK/eIF2α/ATF4, IRE1α/XBP1 and the ATF6. Further studies are however required to fully elucidate the role of ER stress in CD pathogenesis and to test the hypothesis by which ER stress might represent a new therapeutic target in CD treatment.

Importantly, our analysis indicates no major effects by GDF per se, since the basal expression of all evaluated markers is equal in mice fed with a standard vs gluten free diet (Supplementary Figure S3).

Significantly, our data show that probiotics, although not interfering with the effects of active gliadin peptides on intestinal epithelial CFTR, have a beneficial effect thus inhibiting gut inflammation associated with CD. Moreover, our results also indicate a different effect of probiotics, at molecular level, depending on the specific formulation. However, further studies are required to fully understand the molecular mechanisms by which probiotics inhibit the gliadin induced ER stress in CD conditions.

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Figure S1: Schematic representation of the experimental procedure, Figure S2: Caco-2 treatment w/o refresh, Figure S3: Gliadin Free vs Standard Diet, Table S1: Primers sequence.
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5. References


