



# Influence of Intestinal Microbiota in Celiac Disease Pathogenesis and Risk

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Presented by:  
**Marta Olivares Sevilla**

Supervised by:  
**Dr. Yolanda Sanz Herranz**

November 2015







UNIVERSITAT  
POLITÈCNICA  
DE VALÈNCIA



**CSIC**  
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

**Instituto de Agroquímica y Tecnología de Alimentos**

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Dra. Yolanda Sanz Herranz, Profesora de Investigación del Consejo Superior de Investigaciones Científicas en el Instituto de Agroquímica y Tecnología de Alimentos

Hace constar que:

La memoria titulada “Influence of Intestinal Microbiota in Celiac Disease Pathogenesis and Risk” que presenta Marta Olivares Sevilla para optar al grado de Doctor por la Universidad Politècnica de Valencia, ha sido realizada en el del Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC) bajo su dirección y que reúne las condiciones para ser defendida por su autora.

Valencia, 28 de Octubre de 2015

Fdo. Yolanda Sanz Herranz



A mis padres y mi hermana





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# ABSTRACT

Celiac disease (CD) is a chronic enteropathy triggered by cereal gluten proteins in genetically predisposed individuals. The etiology is strongly associated with the genes of the human leukocyte antigen (HLA) encoding the DQ2/DQ8 molecules. Most CD patients carry this genotype but this is also present in the 40% of the general population and only a small percentage develops the disease. Thus, the HLA-DQ genotype is necessary but not solely responsible for the disease development. Gluten is the main environmental trigger but its intake neither fully explains the onset nor its clinical manifestations. Other environmental factors (e.g. early feeding practices, infections, intestinal microbiota) have been associated with the risk of developing CD.

The only treatment for CD patients is the adherence to a gluten free diet (GFD), but the compliance with this dietary strategy is complicated because gluten is present in many foods. Therefore, the identification of modifiable environmental factors that contribute to CD onset is critical for the development of strategies to reduce its incidence.

Observational studies in CD patients revealed imbalances in the intestinal microbiota which could contribute to the pathogenesis of the disease. It has been proposed that these imbalances are not only a secondary consequence of the disease but could also be a predisposing factor. To understand whether gut microbiota imbalances play a role in CD onset and pathogenesis, *in vitro*, animal and human prospective and intervention studies have been conducted in the context of the present PhD Thesis.

The global aim of this Thesis has been to improve the understanding of the role played by intestinal microbiota in the pathogenesis and risk of CD, and the possibilities of contributing to disease prevention and treatment by modulating gut

microbiota composition. **Chapter 1** includes two *in vitro* studies investigating the influence of components of the gut microbiota (bifidobacteria and enterobacteria) on the maturation and functions of immunocompetent cells (dendritic cells), and on gluten toxicity in the intestinal epithelium (Caco-2 cells). We have observed that some *Bifidobacterium* strains are able to reduce the activation of dendritic cells and ameliorate the toxicity of gluten on intestinal epithelial cells.

In **Chapter 2** the effects of the administration of *Bifidobacterium longum* CECT 7347 was evaluated in an *in vivo* model of gluten induced enteropathy in newborn rats, resulting in a reduced proinflammatory cytokine production in the small intestine and CD4<sup>+</sup>T cell numbers in peripheral blood.

**Chapter 3** includes two observational studies in humans to unravel whether breast-feeding and human milk composition and/or the host genotype (HLA-DQ) are related to the microbiota, thereby influencing the later development of CD. We concluded that both factors may contribute to the early intestinal colonization of the infant, influencing the *Bifidobacterium* spp. numbers. Human milk composition also varies in CD and non-CD mothers, modifying the supply of bifidobacteria and protective immune factors to the offspring.

Finally, in **Chapter 4** we have studied the potential beneficial effects of the administration of *B. longum* CECT 7347 in addition to the GFD to children with newly diagnosed CD. This study demonstrates that the bifidobacteria slightly reduces serum inflammatory markers and restored the gut microbiota composition.

## RESUMEN

La enfermedad celíaca (EC) es una enteropatía crónica de carácter autoinmune que sufren individuos genéticamente predispuestos tras la ingesta de gluten. La etiología está asociada con los genes del sistema “Antígeno Leucocitario Humano” (HLA) que codifican las moléculas DQ2/DQ8. Los pacientes con EC presentan este genotipo, sin embargo este también está presente en ~40% de la población general y sólo un pequeño porcentaje desarrolla la enfermedad. Por lo tanto, el genotipo HLA-DQ resulta necesario pero no suficiente para que se desarrolle la enfermedad. El gluten es el principal desencadenante pero su ingesta tampoco explica su desarrollo ni sus manifestaciones clínicas. Otros factores ambientales (p.e. la lactancia, infecciones, microbiota intestinal) se han asociado con el riesgo de desarrollar la EC.

El único tratamiento para los pacientes celíacos es el seguimiento de una dieta exenta de gluten (DEG), sin embargo su cumplimiento es complicado debido a que el gluten está presente en la mayoría de los alimentos procesados. Por ello, la identificación de factores ambientales modificables que contribuyan al desarrollo de la enfermedad, resulta fundamental para desarrollar estrategias que permitan reducir su incidencia.

Estudios observacionales realizados en pacientes con la EC, han demostrado la existencia de desequilibrios en su microbiota intestinal, que podrían contribuir a la patogénesis de la enfermedad. Se ha propuesto que estos desequilibrios no son sólo una consecuencia secundaria de la EC, sino que podrían ser un factor predisponente. Para entender si la microbiota está implicada en el desarrollo y patogénesis de la EC, en la presente Tesis se han desarrollado estudios *in vitro*, con animales y estudios prospectivos y de intervención en humanos.

El objetivo de esta Tesis ha sido avanzar en el conocimiento de la función que la microbiota intestinal desempeña en la patogénesis de la EC, así como, acerca de las posibilidades de tratar o prevenir esta enfermedad mediante la modulación de

la composición de la microbiota intestinal.

El **Capítulo 1** incluye dos estudios *in vitro* en los que se ha estudiado la influencia de componentes de la microbiota intestinal (bifidobacterias y enterobacterias) en la maduración y las funciones de células inmunocompetentes (células dendríticas), y en la toxicidad del gluten en el epitelio intestinal (células Caco-2). Hemos observado que algunas cepas de *Bifidobacterium* son capaces de reducir la activación de las células dendríticas y de reducir la toxicidad del gluten sobre el epitelio intestinal.

El **Capítulo 2** incluye el estudio de los efectos de la administración de *B. longum* CECT 7347 en un modelo de enteropatía inducida por gluten en ratas recién nacidas, observándose una reducción de citoquinas proinflamatorias en el intestino y de células T CD4<sup>+</sup> en sangre periférica.

El **Capítulo 3** incluye dos estudios observaciones en humanos en los que se ha investigado si la lactancia y composición de la leche materna y/o el genotipo (HLA-DQ) están relacionados con la microbiota y, si así, podrían influir en el desarrollo de la EC. Concluimos que ambos factores contribuyen a la colonización intestinal del niño en los primeros meses de vida, afectando especialmente al número de *Bifidobacterium* spp.. La composición de la leche materna varía entre madres celíacas y sanas, lo que podría modificar el aporte de bifidobacterias y factores inmunológicos protectores al lactante.

Por último, el **Capítulo 4** incluye el estudio del potencial efecto beneficioso de la administración de *B. longum* CECT 7347 junto con la DEG en niños recién diagnosticados de EC. Este estudio demuestra que la bifidobacteria reduce ligeramente los marcadores inflamatorios en sangre periférica y contribuye a restablecer la composición de la microbiota intestinal.

## RESUM

La malaltia celíaca (MC) és una enteropatia crònica provocada per les proteïnes del gluten de cereals en individus predisposats genèticament. L'etiologia està fortament associada amb els gens de l'antigen leucocitari humà (HLA). Pacients amb MC són portadors d'aquest genotip però també està present en aproximadament el 40% de la població general i només un petit percentatge (1-3%) desenvolupa la malaltia. Per tant, HLA-DQ genotip és necessària, però no l'únic responsable. El gluten és el principal desencadenant ambiental però la seva ingesta no explica completament l'inici ni les seves manifestacions clíniques. Altres factors ambientals, com la microbiota intestinal, s'han associat amb el risc de desenvolupar la MC.

Els estudis observacionals en pacients amb MC van revelar desequilibris en la microbiota intestinal que podrien contribuir a la patogènesi de la malaltia. S'ha proposat que aquests desequilibris no només són una conseqüència secundària de la malaltia, si no que també podrien ser un factor de predisposició. Per entendre si els desequilibris de la microbiota intestinal podrien tenir un paper en l'aparició de MC, un estudi prospectiu amb el gen en risc la família està en marxa.

L'únic tractament per als pacients amb MC és l'adherència a una dieta lliure de gluten, però el seu compliment és complicat a causa del gluten present en molts aliments. La identificació de factors ambientals modificables que contribueixen a l'aparició de CD és fonamental per a les estratègies de desenvolupament que porten a una reducció de la incidència. Aquest pot ser el cas per als components de la microbiota intestinal, l'adquisició podria ser modulada per factors ambientals i dietètics. L'objectiu global de la tesi és desentranyar els coneixements actuals sobre el paper exercit per la microbiota intestinal en la patogènesi de l'MC, i les possibilitats de contribuir a la prevenció i tractament de la malaltia mitjançant la modulació de la composició de la microbiota intestinal.

En el **Capítol 1** hem estudiat l'ús de models *in vitro* de la influència de la microbiota intestinal durant la maduració i funcions del sistema immunològic (cèl·lules

dendrítiques), i les interaccions entre el gluten i l'intestí i la resposta de l'epiteli intestinal resultant d'aquesta interacció. Hem observat que algunes cepes de *Bifidobacterium* són capaç de reduir l'activació del sistema immune i millorar la resposta nociva de l'epiteli intestinal a l'estimulació amb gluten.

En el **Capítol 2** hem estudiat els efectes de l'administració d'una cepa de *Bifidobacterium* (*B. longum* CECT 7347) per a un model animal de rates nounades. El tractament amb els bacteris es va associar amb una reducció en la producció de citoquines proinflamatòries i la resposta immune de cèl·lules T CD4<sup>+</sup>.

En el **Capítol 3** es va descriure que alguns genotipus (HLA-DQ genotip) i factors ambientals (llet materna) influeixen en la colonització intestinal primerenca, especialment en *Bifidobacterium* spp., que poden influir en l'aparició de MC més tard.

Finalment, en el **Capítol 4** s'ha estudiat l'efecte probiòtic de l'administració de *B. longum* CECT 7347 en xiquets que acaben de ser diagnosticats d'MC i el seu paper en el restabliment de la salut intestinal.



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# I - INTRODUCTION

## 1-Celiac disease

### 1.1-Historical introduction

Celiac disease (CD) likely appeared with one of the first environmental changes associated with human civilization, the development of the agriculture and the introduction of cereals in the human diet. In the first century of Common Era (C.E.) Cappadoce provided the first description of the common manifestation of the disease (chronic diarrhea, abdominal distension and cachexia) and used the term “Koliakos” to name the subjects suffering from this disorder. The etiology of the disease was not discovered until the 1950, when the Dutch pediatrician W. K. Dicke associated the CD symptoms with the consumption of bread and proposed a diet without these cereals to the patients, who showed a clear improvement (1).

In 1957, the development of the Crosby-Kugler capsule to obtain biopsies from the small bowel mucosa, allowed M. Shiner studying duodenal biopsies and demonstrating the typical villous atrophy that characterizes the disease (2). Then, A. Ferguson described the massive infiltration of the small intestinal epithelium by lymphocytes and helped to complete the histological picture of CD (3). In the early 1960, family studies showed the contribution of predisposing genetic factors (4); and 10 years later, new diagnostic tools were developed, based on the detection of circulating immunoglobulin (Ig)-G and IgA antibodies against gluten and the autoantigen tissue transglutaminase (tTG) that underscored the immune-mediated nature of the disease (5). The identification of the human leukocyte antigen (HLA)-DQ2 and HLA-DQ8 as the major predisposing factors (6, 7) and their role in the development of an intestinal inflammatory CD4<sup>+</sup> T lymphocyte response to gluten was crucial to establish the link between the trigger environmental factor and the major predisposing genes (8).

Despite the huge advances in the understanding of CD pathogenesis, many piec-

es of the puzzle remain to be assembled. It is unclear why only a small subset of individuals with the HLA genotype develops the disease and which are the roles of different environmental factors in the onset of the disease. It also remains to be unraveled why the incidence of CD is globally increasing, a phenomenon that cannot be solely explained by improvements in the diagnosis. It is also unclear why some subjects develop CD very early in infancy after the first exposures to gluten; meanwhile others do so lately in the adulthood.

### **1.2-Definition and classification of celiac disease**

The members of the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) group in the year 2012 established the new guidelines for the diagnostic criteria of CD (9). The working group decided to define CD as an immune-mediated systemic disorder elicited by gluten in genetically susceptible individuals and characterized by the presence of a variable combination of gluten-dependent clinical manifestations, CD-specific antibodies, HLA-DQ2 or HLA-DQ8 haplotypes, and enteropathy. CD specific antibodies comprise autoantibodies against tissue transglutaminase 2 (tTG-2), endomysial antibodies (EMA), and antibodies against deamidated forms of gliadin peptides. In these guidelines, it is also indicated that intestinal biopsy may be omitted in symptomatic children with high level of serological markers and HLA-DQ2 or HLA-DQ8 positive. In all other cases, the intestinal biopsy is required in order to avoid misdiagnosis (9).

The ESPGHAN group also reviewed the classifications of CD and distinguished two forms of clinical manifestation: with “gastrointestinal symptoms and signs” and with “extra-intestinal symptoms and signs”. In the first form symptoms such as chronic, diarrhea, abdominal pain, or distension were considered; while the second manifestation included symptoms such as anemia, neuropathy, decreased bone density, or increased risk of fractures. Also, three specific denominations of the clinical manifestations of the disease were established when the defined symptomatology is not sufficient:

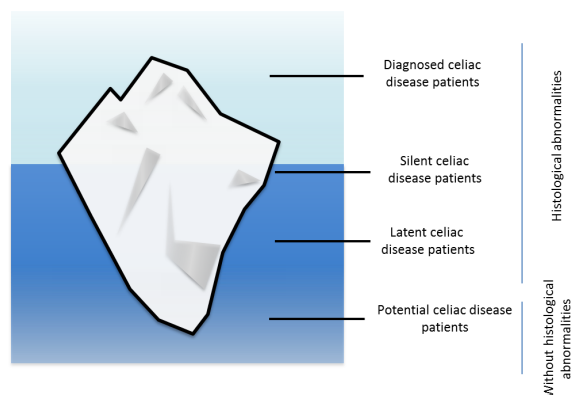
Silent CD is defined as the presence of positive CD-specific antibodies, HLA-

DQ2/DQ8 genotype, and small-bowel biopsy findings that are compatible with CD but without sufficient symptoms and signs to warrant clinical suspicion of CD.

Latent CD is defined by the presence of compatible HLA-DQ2/DQ8 genotype but without enteropathy in a patient who has had a gluten-dependent enteropathy at some point in his or her life. The patient may or may not have symptoms and may or may not have CD-specific antibodies.

Potential CD is defined by the presence of CD-specific antibodies and compatible HLA-DQ2/DQ8 genotype but without histological abnormalities in duodenal biopsies. The patient may or may not have symptoms and signs and may or may not develop a gluten-dependent enteropathy later.

Originally, CD was thought to affect to almost exclusively white Europeans, but now it is known to be widely distributed worldwide and seems to have followed the mankind wheat consumption and the migratory flows (10). The prevalence of the disease ranks between 1 to 3% of the general population (11, 12), even though the existence of the sub-clinical forms described above makes difficult the diagnosis and underestimates the incidence, often represented by an iceberg in which the no symptomatic cases are underwater (**Figure 1**). Some estimations indicate that only 1 of every 5-9 affected individuals is correctly diagnosed and



**Figure 1:** The clinical presentation of CD is often described as an iceberg model. The tip of the iceberg constitutes the diagnosed fraction of CD patients and the part underwater those who are undiagnosed.

treated.

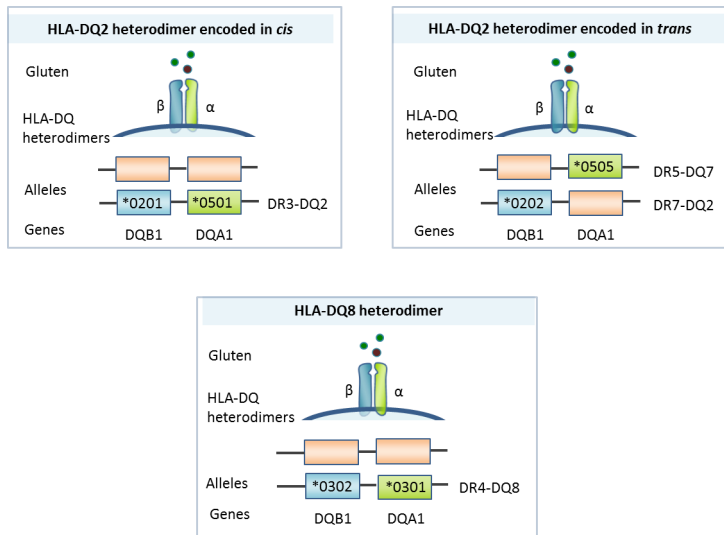
### 1.3-Pathogenesis of celiac disease

CD is a multifactorial disorder involving genetic and several environmental factors. The disease is strongly associated with the HLA genes that codify the expression of the HLA-DQ2/DQ8 heterodimers in the surface of the antigen presenting cells (APC). Most CD patients present this genotype; however this is also present in ~40% of the general population and only a small percentage (1-3%) finally develops the disease (11, 12). Thus, the genotype HLA-DQ2/DQ8 is needed but it's no sufficient to explain the onset of CD (13). Currently, non-HLA genes are known to be involved as described below. Gluten intake is the environmental factor responsible for the manifestation of the disease; however, the number of patients diagnosed during the adulthood after many years of gluten exposure is increasing demonstrating that gluten intake may not be the only trigger (14). Epidemiological studies indicate that other factors may increase or reduce the risk of developing CD. The amount and timing of gluten introduction to the infant diet, the type of delivery, the milk feeding practices, the incidence of infections and the intestinal microbiota composition have been proposed to influence the CD risk. However, no conclusive results have been obtained so far (reviewed in 15).

#### 1.3.1-Genetics

Different studies in siblings have demonstrated the strong genetic component of CD, showing a concordance of around 80% in monozygotic twins *versus* less than 20% among dizygotic twins, and 10% in first degree relatives of CD patients (16-18). HLA-DQ has been the locus more widely studied in the context of CD. The HLA-DQ genes encode molecules expressed on the surface of the APCs involved in the recognition of the gluten peptides. It has been described that approximately 90-95% of CD patients carry HLA-DQ2 heterodimers formed by a  $\beta$ -chain encoded by HLA-DQB1\*02 allele (either\*0201 or \*0202) and an  $\alpha$ -chain encoded by HLA-DQA1\*05 allele. HLA-DQB1\*02 and HLA-DQA1\*05 alleles can be inherited in *cis* (when both are present on one parental chromosome), or in

*trans* (when the  $\alpha$ - and  $\beta$ -chains are encoded by chromosomes from each parent). The remaining percentage (5-10%) of patients express the HLA-DQ8 heterodimer formed by  $\beta$  and  $\alpha$ -chains encoded by HLA-DQB1\*0302 allele and HLA DQA1\*0301 allele, respectively. HLA-DQ2 and HLA-DQ8 molecules share peptides binding motifs characterized by a preference for hydrophobic and negatively charged amino acids, such as the deaminated gluten peptides (**Figure 2**).



**Figure 2:** Representation of genes, combination of alleles (haplotypes) and codified HLA-DQ or HLA-DQ8 heterodimers ( $\alpha$  and  $\beta$  chains). Celiac disease patients who are DR3 or DR5/DR7 express the same molecule: HLA-DQ2. The genes DQB1 and DQA1 can be inherited in cis (in the same parental chromosome), or in trans (when the  $\alpha$ - and  $\beta$ -chains are encoded by the corresponding chromosome from each parent). CD patients who are DR4 express the molecule HLA-DQ8. Adapted from Dieli-Crimi *et al.*, 2015 (19).

Over the last decades, different genetic studies have been performed in order to reveal all the genetic factors involved in CD. Nowadays, CD is well established as a polygenic disease with a complex non-Mendelian pattern of inheritance. So far, 58 genetic variants located within 36 non-HLA regions have also been associated with the susceptibility to suffer from CD, being located only 3 of these variants on protein coding regions and the remaining variants located in non-coding regulatory regions and intergenic regions of unknown function. Thus, CD onset is influenced by the accumulation of risk due to HLA and non-HLA genes together

with different environmental factors (reviewed in 19).

### **1.3.2-Gluten as environmental trigger**

In 1950, wheat was described as causal agent (antigen) of the CD (1). Then, barley and rye grains were described as toxic for CD patients. Still there are controversies about the possible toxic effects of oats (reviewed in 20). Because of the ubiquity of wheat in the diet and the controversy with oats, both cereals have been the most widely studied. The gluten fraction soluble in alcohol and characterized by having a high content in proline is denominated prolamin; while the fraction insoluble in alcohol is the glutenin. Prolamin proteins are named more specifically based on the cereal where they come from. Thereby in wheat, barley and rye, the prolamins are called gliadin, hordein and secalin, respectively. Gliadin, hordein and secalin have a high proline and glutamine content. These particular amino acid residues confer to these proteins a partial resistance to the proteolytic digestion since gastric, pancreatic and brush border enzymes are deficient in prolyl-endopeptidases with the consequent accumulation of immunogenic peptides in the intestinal lumen (21). However, the relatively poor digestion of these proteins alone is not sufficient to cause CD, and indeed there is no known difference between healthy individuals and susceptible CD subjects in their ability to digest gluten proteins (22).

In the small intestine gliadin peptides interact with the CXCR3 receptor that leads to the release of zonulin (a regulatory protein of the tight junctions between enterocytes) that, in turn, increases intestinal permeability. This mechanism may facilitate the passage of gluten peptides by the paracellular pathway to the lamina propria (23). Besides, gluten peptides can also cross the intestinal barrier using the ability of enterocytes to capture molecules from the luminal space, through the so-called transcellular route via transcytosis. For instance, the translocation of the 33-mer peptide (p57-89 from the  $\alpha$ -gliadin) can occur by transcytosis regulated by interferon (IFN)- $\gamma$ . This uptake has been observed to be higher in untreated CD than in controls and CD patients on a GFD (24). Moreover other peptides, such as the p31-49 from the  $\alpha$ -gliadin, can be transported intact through an abnormal retrotranscytosis process mediated by secretory immuno-



globulin A (sIgA) and the CD71 receptor (25). In normal conditions, this receptor plays a major role in iron uptake and metabolism, and it is only expressed in the basolateral membrane of enterocytes and mainly in the crypts. In active CD, CD71 is massively upregulated and expressed in the apical membrane of enterocytes where the sIgA1-gliadin complexes present in the lumen can bind and translocate intact into the lamina propria (25). This mechanism, seen in patients with active CD but not in controls or treated patients, represents an exception to the well-characterized protective effect of sIgA in the mucosal surface, where it binds antigens and facilitates their secretion in faeces (reviewed in 26). Interestingly, some authors have proposed that the over-expression of CD71 receptor is probably due to iron deficiency, which is frequent in CD patients (27).

In recent years the role of the alterations in the intestinal permeability in CD is attracting attention as may be one of the first events that precede the onset of the disease. Nevertheless, gluten is also known to activate an inflammatory response with production of cytokines that cause gut barrier disruption, creating a vicious-circle where both mucosal inflammation and the leaky gut influence one another as explained below.

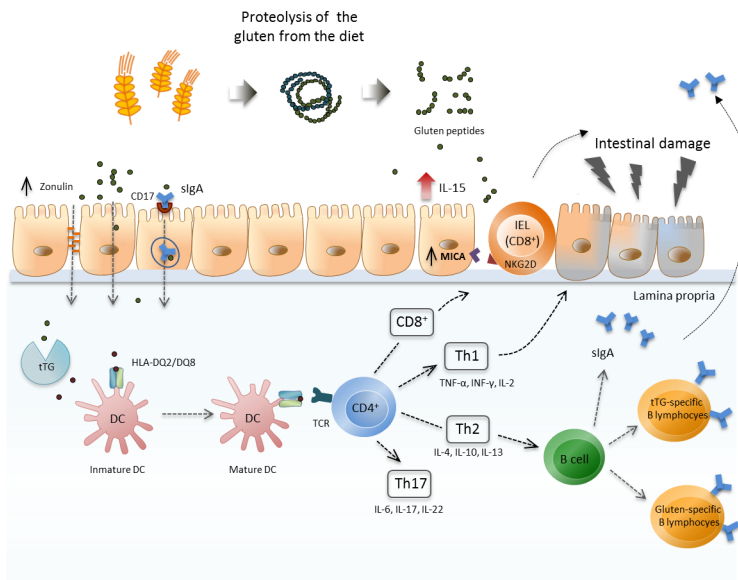
The patten and timing of gluten introduction into the infant's diet has been considered as one of the critical factors that determines CD risk. In this regard, two independent intervention studies have investigated whether there is a window of opportunity to introduce gluten into the diet that confers protection against CD in genetically predisposed individuals. One randomized double-blinded intervention study (PreventCD) in which infants at genetic risk of developing CD (HLA-DQ positive) (n=944) received a small dose of gluten or a placebo daily between 4-6 months of age, showed no differences between groups in the prevalence of CD at 3 years of age (28). The second study (CELIPREV) found that the introduction of gluten at 12 months (compared to 6 months) in infants genetically predisposed to develop CD (n=832) may delay the onset of the disease but does not modified its incidence at 5 years of age (29).

Regarding CD in the adulthood, a recent study in mice with altered stress-related behavior and premature immune senescence suggests that aging and an

altered-stress response play a role in the CD susceptibility through the altered immune response to gluten and intestinal bacteria (30).

### 1.3.3-Immunopathogenesis

The immunopathogenic process of CD involves both innate (gluten direct effect on the epithelium and innate immune cells) and adaptive immunity (through the lamina propria  $CD4^+$  and  $CD8^+$  T lymphocytes) (**Figure 3**).



**Figure 3:** Schematic representation of CD pathogenesis. Some gluten peptides cross the intestinal epithelium and can be deamidated by the tissue transglutaminase (tTG), which increases their ability to bind the HLA-DQ2/DQ8 molecules of APCs and to trigger an adaptive immune response, involving Th1, Th2 and Th17 cells that lead to the release of pro-inflammatory cytokines and the production of CD antibodies; other gluten peptides activate an innate immune response by interacting with epithelial cells and APCs and, thus, triggering the production of the pro-inflammatory cytokines. In particular, IL-15 increases the expression of the MICA molecule in epithelial cell surface and triggers activation of intraepithelial lymphocytes through engagement of NKG2D, leading to an innate-like cytotoxicity toward epithelial cells and enhanced  $CD8^+$  T cell-mediated adaptive response, contributing to villous atrophy. Adapted from Olivares and Sanz, 2015 (31).

Regarding the adaptive immune response, once gluten peptides reach the lamina propria through the different mechanisms described above, the enzyme tTG de-

aminates the glutamine residues (positively charged) to glutamic acid (negatively charged). The HLA-DQ2 and HLA-DQ8 molecules expressed in the surface of the APCs (basically dendritic cells) have in their peptide binding groove several “pockets” that favor binding of negatively charged residues. Thus, tTG significantly increases the affinity and the capture of gliadin peptides, constituting a key factor in the CD pathogenesis (reviewed in 32).

APCs are very efficient at internalizing gluten peptides and, then, displaying some of the fragments on their surface. The specific gluten epitopes that can be displayed by a person's APCs depend on the set of antigen-presenting molecules encoded by that person's genome within the HLA locus. The union HLA-DQ-gluten on the surface of the APCs is recognized by the T cell receptor (TCR) presented on the surface of the CD4<sup>+</sup>T lymphocytes, triggering the activation of CD4<sup>+</sup>T lymphocytes into the pro-inflammatory T helper (Th) 1 profile with the release of mainly IFN- $\gamma$ , as well as tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-2; and also the Th17 response with the release of IL-6, IL-17A and IL-22 (reviewed in 32).

These cytokines cause tissue damage and the loss of the normal intestinal architecture through the infiltration of CD8<sup>+</sup> T lymphocytes and the release of metalloproteinases (reviewed in 32). Activated CD4<sup>+</sup> T lymphocytes can also drive a Th2-type response that leads to the release of other cytokines (IL-4, IL-10, IL-13). The Th2-type response leads to clonal expansion of differentiated B lymphocytes into plasma cells (effector B cells) that produce anti-transglutaminase and anti-gliadin antibodies (33) which induce changes in the cytoskeleton and the structure of the enterocytes. Overall, this causes the development of typical lesions of CD, with crypt hyperplasia and villous atrophy (reviewed in 32).

Furthermore, it has been recently described that certain peptides present toxic actions on the epithelium not related to the adaptive response mediated by T lymphocytes. These peptides induce the release of IL-15 by enterocytes, dendritic cells and macrophages. In turn, this cytokine induces the expansion of intraepithelial lymphocytes (IELs) and the acquisition of a natural killer cell phenotype. IL-15 also induces the expression of the stress MICA molecule (MHC class I

chain-related molecule A) in the epithelial cells. Finally, the binding of the MICA to the NKG2D receptors on the lymphocytes with natural-killer activity (CD8<sup>+</sup> IELs) promotes the destruction of enterocytes (34).

#### **1.4-Therapeutic strategies**

Life-long gluten exclusion from the diet is the only available treatment for CD patients. In all cases, CD patients should exclude wheat, barley, rye and oats and derivatives from the diet. After two weeks of adherence to a gluten free diet (GFD), an improvement in symptoms is observed; then, the serological normalization appears between 6-12 months and the recovery of the intestinal villi around two years after the adherence to the diet depending on the severity of the disease and the age (reviewed in 35).

However, this dietary restriction often affects the social life of CD patients, limits the variety of food choices in their diets and it is difficult to maintain because gluten is present in most of the processed foods. The progress in the understanding of the molecular and cellular events involved in the abnormal response to gluten has led to the development of alternative or complementary therapeutic strategies to the GFD. Briefly, some of the alternatives that are being investigated are described below.

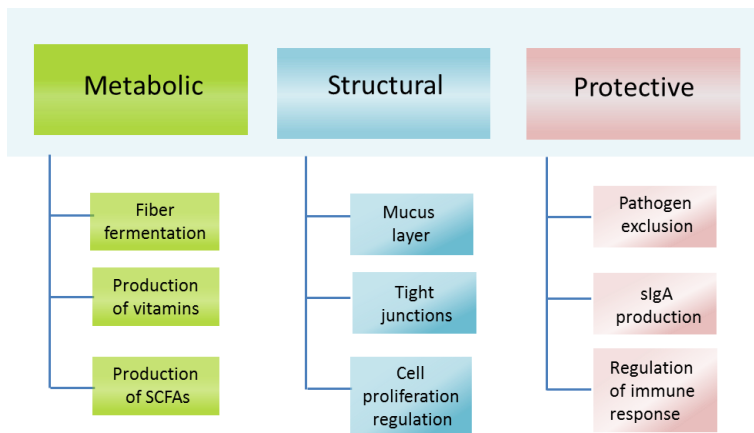
- Prolyl-endopeptidases and glutamine-endopeptidases from bacterial and fungi sources to break down the resistant immunogenic gluten peptides and detoxify gluten (36).
- Inhibition of the tTG activity to reduce the affinity of the gluten peptides for the HLA-DQ2/DQ8 molecules (37)
- Blockade the HLA-DQ2/DQ8 molecules using synthetic peptides agonists that compete with gluten peptides for the binding sites of the HLA-DQ molecules (38).
- Immunomodulation by the induction of anti-inflammatory cytokines (IL-10) (39) or by the administration of antagonists of pro-inflammatory cytokines such

as IL-15 (40) or TNF- $\alpha$  and IL-18 (41).

## 2-Celiac disease and microbiota

### 2.1-The intestinal ecosystem

It has been estimated that the intestine harbors  $10^{13}$  to  $10^{14}$  microorganisms (42), which means that the bacterial cells in the intestine are ten times more numerous than the total number of human body cells. Additionally, the collective genome of the microbiota, named microbiome, contains at least 100-fold more genes than the complete human genome (42, 43). The intestinal microbiota is considered to play numerous functions in the human body, including metabolic, protective and structural functions (**Figure 4**). Despite the important role that the intestinal microbiota seems to play in human health, it is not known yet which are the components of the microbiota that are responsible for specific conditions and those that provide protection from disease. Cultivation techniques have limited resolution because only a small fraction (20-40%) of the intestinal bacterial species is able to be grown using culture-based techniques (45, 46). In this context, the development and use of high-throughput DNA sequencing platforms applied to 16S rRNA gene and metagenomic analyses have rapidly increased the understanding of the taxonomy and diversity of the human intestinal microbiota and



**Figure 4:** Main functions of the intestinal microbiota impacting on the human health. Adapted from Grenham *et al.*, 2011 (44).

their relationship with health and disease.

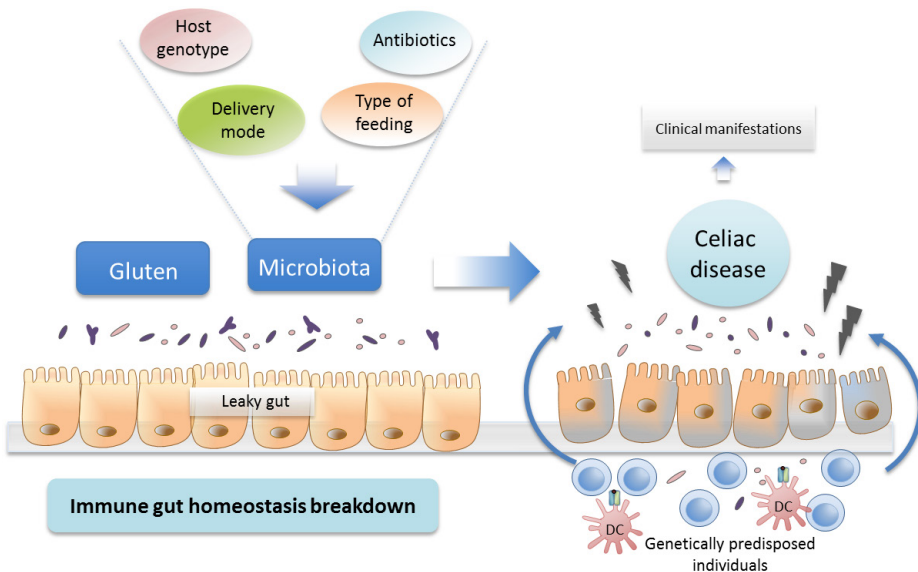
16S rRNA and metagenomic studies have revealed that the majority of gut microbiota sequences belong to the Bacteria, which is the predominant kingdom in the human adult gut (45, 46). Despite the complexity of the human intestinal ecosystem, the majority of the bacteria belong to 6 dominating phyla Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, Verrucomicrobia, and Fusobacteria. Actinobacteria is the most abundant phylum in infants because of the predominant colonization by the genus *Bifidobacterium* in the first stages of life (47, 48), meanwhile Firmicutes and Bacteroidetes dominate the intestinal microbiota of the adult population (45, 49).

## 2.2-Early factors influencing gut microbiota and CD risk

The primary colonization of the intestinal tract begins at birth with the acquisition of microbes from the environment, mainly from the maternal vagina and the skin. It is a dynamic process that involves interactions of co-occurrence and exclusion between intestinal bacteria, reflecting life events of the newborn and undergoing changes until the first 2-3 years of age when the microbiome starts to converge toward an adult-like profile (47, 50). The intestinal colonization process leads to the acquisition and establishment of a protective microbiota that could modulate the risk of developing immune-mediated diseases in adulthood (51). This influence is mediated by the interactions between the gut microbiota, the diet and immune system, which are crucial for the development of tolerance towards harmless antigens from the diet and the commensal microbiota and appropriate defenses against pathogens.

Perinatal and early postnatal environmental factors influencing the microbiota composition have been associated with CD susceptibility (**Figure 5**). The greater risk of children born by caesarean section of developing CD (53) might be related to the delay in intestinal colonization by bifidobacteria and the reduced bacterial diversity observed in caesarean-born compared to naturally-delivered infants (54). Longer breast-feeding and particularly maintenance of breast-feeding when gluten is introduced seem to reduce the risk of developing CD or, at least, delay

its onset in most case-control retrospective studies included in the meta-analysis by Akobeng *et al.* (55). Also, feeding practices involving the gradual introduction of gluten simultaneous to breastfeeding were proposed as the protective factor responsible for reducing CD prevalence in one birth cohort compared to the “Swedish CD epidemic” cohort (56). However, other prospective epidemiological and intervention studies failed to find a protective effect of breast-feeding in either CD autoimmunity or biopsy proven CD (57, 58). This inconsistency could be due to the lack of control of other confounding variables that have not been systematically investigated so far.



**Figure 5:** Proposed model for CD pathogenesis. Specific host genetic make-up and environmental factors could promote the colonization of pathobionts and reduce symbionts, thus leading to dysbiosis. Dysbiosis may contribute to disrupting the immune homeostasis and gut integrity, thereby favoring CD onset or aggravating the pathogenesis. Adapted from Cenit *et al.*, 2015 (52).

Duration of breast-feeding could be associated with a reduced or delayed exposure of the newborn to dietary gluten, which might contribute to the protective effect of breast milk. Plausibly bioactive breast milk components may also be involved in the potential protective effect of breast-feeding on CD development. For instance, breast milk seems to be a main source of bacteria for the infant’s gut

(59, 60) and of human milk oligosaccharides (HMOs), which promote colonization of *Bifidobacterium* spp.. This could explain the differences observed between the intestinal microbiota of breast-fed and formula-fed infants (61-63). Besides human milk provides many others bioactive substances involved in passive immune protection and in immunological development of the neonate (64).

On the other hand, a positive association between antibiotics use and subsequent development of CD has been found in a case-control study when the histopathology data of nearly 3,000 subjects with CD were linked to the Swedish Prescribed Drug Register (65). Based on these results, the authors concluded that antibiotics intake may increase the risk of suffering CD due to their profound impact on intestinal microbiota. However, this association was not found when the exposure to antibiotics during pregnancy and the development of CD in the offspring was investigated (66), although this last study was most probably underpowered for this purpose.

The host genotype has also been described to influence the composition of the human gut microbiota and modulate the phenotype (67). In the case of CD, a prospective study in a cohort of 164 infants with a family history of the disease reported associations between genetic risk (HLA-DQ) and alterations in the intestinal microbiota composition (68-70). The HLA-DQ2/DQ8 genotype and the type of feeding (maternal or formula) influenced in conjunction the intestinal colonization analyzed by fluorescence *in situ* hybridization (FISH), real time PCR and denaturing gradient gel electrophoresis (DGGE) techniques (68-70). In addition, specific decreases in *Bifidobacterium* spp. and *B. longum* and increases *Staphylococcus* spp. were associated with higher genetic risk of developing CD irrespective of milk-feeding type (69).

In conclusion a number of epidemiological studies indicate that several perinatal and early postnatal factors influence the intestinal microbiota composition and participate in conjunction to modulate CD risk. However, there are no large prospective studies revealing how the differences in the intestinal microbiota ac-



quisition in early life might ultimately protect or contribute to the development of CD.

### **2.3-Influence of intestinal microbiota in CD pathogenesis**

Several observational studies in children and adults with CD have shown alterations in the intestinal microbiota composition compared to control subjects (**Table 1**). Our studies using molecular quantitative methods, such as FISH and quantitative PCR, found reduced numbers of *Bifidobacterium* spp. and *B. longum* and increased numbers of *Bacteroides* spp. in stools and duodenal biopsies of CD patients untreated and treated with a GFD compared to control subjects (71, 72). Also enterobacteria and staphylococci numbers were higher in untreated CD patients than in controls, but these differences were almost restored in CD subjects on a long-term GFD (72). Likewise, other studies in children reported increased prevalence of *Bacteroides vulgatus* and *Escherichia coli* in CD biopsies before and after the GFD by temporal temperature gradient gel electrophoresis (TTGE) compared to controls (73) and lower numbers of *Lactobacillus* and *Bifidobacterium* and higher numbers of *Bacteroides*, *Staphylococcus* and enterobacteria in stools of children with CD compared to healthy controls (77). Other studies performed by DGGE of the microbiota of adults clustered the dominant microbial communities of healthy individuals together and separated from those of untreated CD patients (76). However, the above study reported an increased prevalence in *Bifidobacterium bifidum* in patients with active CD as opposed to the lower bifidobacteria numbers detected in CD patients in our studies (71, 72, 77) or the absence of differences reported in another study (79). In Sweden, an early study with samples collected in 1985-1996 revealed that rod-shaped bacteria were frequently associated with the mucosa of CD patients; both in the active phase and treated with a GFD, as detected by scanning electron microscopy (SEM) (74). Later, these SEM analyses were complemented with 16S rDNA sequencing to identify the bacterial communities detected in the samples of the Swedish epidemic (1985-1996) and in a new cohort of patients (2004-2007) (75). Only one CD biopsy collected during 2004-2007 contained rod-shaped bacteria in contrast to the frequency described in the samples of the Swedish

Analyzed cohort	Sample	Technique	Results	ref
Children with active CD and symptom-free CD patients on GFD.	Duodenal biopsy	FISH couple with flow cytometry	↑ Proportions of total bacteria and Gram-negative in active CD ↑ <i>Bacteroides</i> and <i>E. coli</i> in active CD and normalized after GFD ↓ <i>Lactobacillus</i> + <i>Bifidobacterium</i> / <i>Bacteroides</i> + <i>E. coli</i> in CD patients	71
Children newly CD diagnosed and after GFD	Faecal and biopsy	Real-time qPCR	↑ <i>Bacteroides</i> and <i>Clostridium leptum</i> in CD patients ↑ <i>E. coli</i> and <i>Staphylococcus</i> in non-treated CD and normalised after GFD ↓ <i>Bifidobacterium</i> in faeces of both groups of CD patients and in biopsies of newly CD	72
Children with CD before and after GFD	Duodenal biopsy	TTGE	+ Number of bands in CD patients + Abundance of <i>Bacteroides vulgatus</i> and <i>E. coli</i> in CD patients	73
Children with CD (untreated, treated or challenge)	Jejuna biopsies	SEM	+ Rod-shaped bacteria associated with the mucosa of CD patients (active and inactive)	74
Children with CD (untreated, treated or challenge)	Small intestine biopsies	SEM 16S rRNA seq.	No differences between CD and controls + Frequency of <i>Atopobium parvulum</i> -like and its clones in treated CD than in untreated + Frequency of <i>Haemophilus</i> in untreated CD than in treated CD	75
Adults with treated and untreated CD	Faecal samples	DGGE	- Diversity of <i>Lactobacillus</i> and <i>Bifidobacterium</i> species in treated CD patients + <i>Bifidobacterium bifidum</i> in untreated CD than in healthy adults	76
Children with CD on GFD	Duodenal biopsy and faecal samples	DGGE Cell cultures	+ Diversity in Eubacteria in duodenal samples of CD + <i>Lactobacillus</i> , <i>Enterococcus</i> and <i>Bifidobacteria</i> levels in faecal samples of controls ↑ Cell counts of <i>Bacteroides</i> , <i>Staphylococcus</i> , <i>Salmonella</i> , <i>Shigella</i> and <i>Klebsiella</i> in CD ↓ Percentage of lactobacilli in controls	77
Healthy population before and after GFD	Faecal samples	FISH Real-time qPCR	↓ <i>Bifidobacterium</i> , <i>C. lituseburensis</i> and <i>F. prausnitzii</i> proportions after GFD by FISH ↓ <i>Bifidobacterium</i> , <i>Lactobacillus</i> and <i>B. longum</i> and ↑ <i>Eritrobacteriaceae</i> and <i>E. coli</i> after GFD by qPCR	78
Children with untreated CD and adults with CD on GFD	Intestinal biopsy	Real-time qPCR	No differences in the amounts or frequencies of the bacteria analyzed ( <i>Bifidobacterium</i> , <i>Bacteroides</i> - <i>Prevotella</i> , <i>Bacteroides</i> , <i>Streptococcus</i> nor <i>Lactobacillus</i> )	79
Children with untreated CD	Intestinal biopsy	PCR couple to IS-pro	No differences neither composition nor diversity	80
Adult CD patients with different symptomatology	Duodenal biopsy	DGGE 16S rRNA sequencing	- Microbial diversity in CD patients with gastrointestinal symptoms than those with dermatitis herpetiformis Proteobacteria characterized the microbiota CD with gastrointestinal symptoms Similarity between CD patients with dermatitis herpetiformis and control characterized by Firmicutes	81

**Table 1:** Summary of studies reporting associations between gut microbiota composition and celiac disease

epidemic, invalidating the initial theory that these bacteria were causative factors of the CD epidemic.

The analysis of metabolites derived from intestinal microbiota activity has also revealed significant differences between treated CD patients and healthy controls and suggests that there is a metabolic signature for the CD microbiome (76, 77). One recent study reported that CD patients with gastrointestinal symptoms had different microbiota composition when compared with controls and patients with dermatitis herpetiformis, suggesting that the microbiota may play a role in the manifestation of the disease (81). Dysbiosis of the gut microbiota has been also associated with persistent gastrointestinal symptoms in treated CD patients (82).

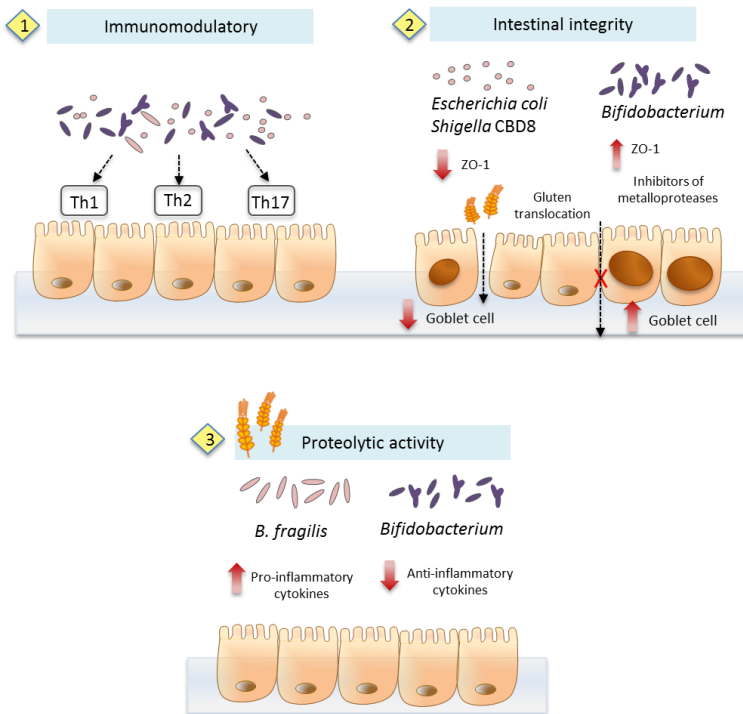
Nevertheless, we should also mention that other authors report no differences in mucosa-associated duodenal microbiome composition and diversity using a 16S-23S rRNA interspacer region-based profiling method (80). There is also lack of consensus and understanding of what constitutes a CD-promoting microbiota. A deeper characterization of the CD microbiota by isolating bacterial strains and analyzing their pathogenic features (83-85) showed that the *E. coli* clones belonging to virulent phylogenetic groups (B2 and D) isolated from untreated and treated CD patients presented a higher number of virulence genes encoding for P fimbriae, capsule K5 and hemolysin than those isolated from healthy controls (83). Also, the abundance of the species *Bacteroides fragilis* coding for metalloproteases was increased in both untreated and treated CD patients, and thus could presumably play a pathogenic role in CD (84). The isolation and identification of clones belonging to the genus *Staphylococcus* also revealed that the species *S. epidermidis* carrying the *mecA* gene (methicillin resistant gene) was more abundant in the CD patients (treated and untreated) than in controls (85).

The only treatment for CD is the adherence to a life-long GFD, which implies important dietary changes. These dietary differences also seem to cause changes in the intestinal microbiota composition and in the immune response to the altered microbiota *in vitro*. In healthy adults the GFD caused shifts in gut microbiota composition, characterized by reduced numbers of *Bifidobacterium* spp., *B.*

*longum* and the *Lactobacillus* group, and increased numbers of *Enterobacteriaceae* and *E. coli* (78). This led to suggest that GFD should be considered as an environmental factor that may contribute to shaping the microbiota composition in treated CD patients (78).

## 2.4-Mechanism of action of intestinal microbiota in CD

The microbiota could contribute to the etiopathogenesis of CD by at least three mechanisms: by mediating host-microbe interactions that influence the immune function and the intestinal barrier; and providing proteolytic activities that influence the generation of immunogenic peptides from gluten (Figure 6).



**Figure 6:** Schematic representation of the potential role of intestinal microbiota .The microbiota could contribute to the etiopathogenesis of CD by (1) the modulation of the immune function, (2) mediating host-microbe interactions which could influence the intestinal barrier, or (3) providing proteolytic activities that influence the generation of toxic and immunogenic peptides from gluten.

1- Gut bacteria present immunomodulatory properties that may exert a harmful or beneficial effect on the host's health. For instance, it has been described that a

mixture of isolated bacteria from CD patients (*Prevotella* spp., *Lachnoanaerobaculum umeaense* and *Actinomyces graevenitzi*) induced IL-17A mRNA expression in *ex vivo* biopsies of intestinal mucosa of CD patients (86). It was hypothesized that those bacteria could modulate the IL-17 response by helping to break-down the gluten tolerance (86). On the other hand and as an opposite potential effect of the intestinal microbiota, *B. longum* CECT 7347 and *B. bifidum* CECT 7365 reduced the inflammatory response (IFN- $\gamma$  and TNF- $\alpha$ ) observed in peripheral blood mononuclear cells (PBMCs) exposed to faeces of CD patients (87).

2- Gut bacteria can also strengthen or damage the intestinal integrity. For instance, CD-triggers (gliadin and IFN- $\gamma$ ) decreased the goblet cell numbers in intestinal loops of inbred Wistar-AVN rats, and the presence of enterobacteria isolated from CD patients (*E. coli* CBL2 and *Shigella* CBD8) aggravated this effect (88). By contrast, *B. bifidum* CECT 7365 increased the number of goblet cells and the production of inhibitors of metalloproteinases, and also reduced gliadin translocation to the lamina propria, which could contribute to gut mucosal protection (88). Changes in tight junctions were measured and meanwhile the exposure to *B. bifidum* CECT 7365 together with gliadin and IFN- $\gamma$  increased ZO-1 levels, the addition of *E. coli* CBL2 reduced ZO-1 levels (88). Other probiotic bacteria such as *L. rhamnosus* GG have shown to contribute *in vitro* to the maintenance of normal intestinal permeability in Caco-2 cells exposed to gliadin (89).

3- Gut microbiota provide proteolytic activities to break-down gluten peptides. As described above, some gluten peptides resist gastrointestinal digestion and disturb the intestinal homeostasis (90). This may facilitate the access of gluten to the lamina propria and their interaction with immune cells. *In vitro* studies indicate that the proteolytic activity of the intestinal microbiota may modify gluten peptides differently, increasing or reducing their toxicity. *B. fragilis* strains isolated from CD patients when incubated with gliadin generated immunogenic peptides that preserved or increased the production of TNF- $\alpha$ , and showed increased ability to permeate through Caco-2 cells (91). Meanwhile, different *Bifidobacterium* spp. and especially *B. longum* CECT 7347 attenuated the pro-inflammatory response of gliadin-derived peptides after the *in vitro* digestion (84).



## II - OBJETIVES

The general objective of this Doctoral Thesis was to study the influence of the intestinal microbiota and specific components on CD pathogenesis and risk.

In order to achieve this main objective, the following specific objectives were proposed:

1. To evaluate the *in vitro* effects of different bifidobacteria and enterobacteria identified in CD patients on the regulation of the production of proinflammatory markers and the proteomic response to gliadins in cell cultures.
2. To evaluate the *in vivo* effect of the administration of a strain of the genus *Bifidobacterium* in an animal model of gliadin induced enteropathy.
3. To investigate the potential role of environmental and genetic factors on breast-milk composition of healthy mothers and mothers with CD, and on the intestinal colonization process in a cohort of healthy children at family risk of developing CD.
4. To evaluate the effect of the administration of a *Bifidobacterium* strain on the intestinal ecosystem and immunological markers in children newly diagnosed with CD.





# III – RESULTS AND DISCUSSION

## Chapter 1

- **Modulation of phenotypic and functional maturation of dendritic cells by intestinal bacteria and gliadin: relevance for celiac disease.**
- **Influence of *Bifidobacterium longum* CECT 7347 and gliadin peptides on intestinal epithelial cell proteome**



# **Modulation of phenotypic and functional maturation of dendritic cells by intestinal bacteria and gliadin: relevance for celiac disease**

G. De Palma<sup>1</sup>, J. Kamanova<sup>2</sup>, J. Cinova<sup>2</sup>, J., M. Olivares<sup>1</sup>, H. Drasarova<sup>2</sup>, L. Tuckova<sup>2</sup> and Y. Sanz

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<sup>1</sup>Microbial Ecology and Nutrition Research Group, Institute of Agrochemistry and Food Technology, National Research Council (IATA-CSIC), Valencia, Spain; <sup>2</sup>Institute of Microbiology, Dept Immunology, Academy of Sciences of the Czech Republic, Prague.



## ABSTRACT

Dendritic cell (DC) maturation and functions are influenced by microbial and environmental stimuli, which could contribute to immune dysfunction. Here, we have investigated the role of enterobacteria (*E. coli* CBL2 and *Shigella* CBD8) isolated from celiac disease (CD) patients, bifidobacteria (*B. longum* CECT 7347 and *B. bifidum* CECT 7365) and gliadins on phenotypic and functional features of monocyte-derived dendritic cells (MDDCs) and in co-culture with Caco-2 cells. The ultimate goal of our study is to understand the roles played by specific components of the gut microbiota in CD. Enterobacteria induced marked alterations in MDDCs morphology, inducing podosome dissolution and dendrites, and activated MDDC adhesion and spreading. Enterobacteria also induced inflammatory cytokine production (IFN- $\gamma$ , TNF- $\alpha$ , IL-12), partially resembling the gliadin-induced Th1-type cytokine profile. *B. longum* CECT 7347 and *B. bifidum* CECT 7365 induced minor MDDCs morphological changes, and activated adhesion and spreading and inflammatory cytokine production to a lesser extent compared to enterobacteria. *B. longum* CECT 7347 also induced lower CD86 and CD40 expression on MDDCs than the two enterobacteria. The aforementioned bifidobacterial strain also reduced gliadin-induced IFN- $\gamma$  production and increased IL-10 secretion when both stimuli were combined. Similar trends were detected for MDDCs co-cultured with Caco-2 cells. *B. longum* CECT 7347 reversed the gliadin-reduced ZO-1 expression in Caco-2 cells. Thus, our results suggest that specific components of the gut microbiota may influence phenotypic and functional maturation of DCs differently, and their interactions with epithelial cells. This could ultimately define the role of DCs in celiac disease progression.

**Keywords:** dendritic cells, celiac disease, bifidobacteria, enterobacteria, gliadin

## **INTRODUCTION**

Celiac disease (CD) is a chronic inflammatory disorder of the small intestine characterized by an aberrant immune response to gluten proteins (gliadin) in genetically predisposed individuals. The disease is caused by the interaction between genetic and environmental factors (1). CD shows a strong association with Human Leukocyte Antigen (HLA)-DQ genes, with most patients carrying a variant of DQ2 or DQ8. The association between HLA and CD is stronger than for other HLA-linked diseases, contributing 53% to disease risk (1). Dietary gluten is the key environmental factor involved in the disease. Gluten proteins are rich in proline and glutamine residues, making them resistant to proteolytic degradation within the gastrointestinal tract and good substrates for the enzyme tissue transglutaminase. This enzyme deamidates glutamine into negatively charged glutamic acid, increasing the affinity of gluten peptides for HLA-DQ2 and HLA-DQ8 molecules of antigen presenting cells (APCs) (2,3). Both innate and adaptive immune responses contribute to triggering mucosal inflammation in the patients (3). CD is described as a Th-1 cell mediated disorder because Th1-type cytokines are overproduced in intestinal CD mucosa (4). Moreover, Th17 cells contribute to the ongoing inflammation in CD (5). IL-21, which is a Th17 cell-derived cytokine

promoting Th17 cell development, is also overproduced in CD. This cytokine is also associated with the local production of IFN- $\gamma$  by CD4+ Th1 cells (6). More recently, the presence of gluten-specific IL17A-producing cells secreting IL-21 and high levels of IL-22 and regulated by TGF $\beta$  through a RORC- and IRF-4-dependent mechanism had been reported in duodenal biopsies of CD patients (7). In active CD patients, gut permeability is also increased and related to dysfunction of the intercellular junction between epithelial cells due to reorganization and changes in expression of tight junction (TJ) related proteins (1).

This pathological process leads to typical histological characteristics of CD, including small intestinal mucosal villous atrophy, crypts hyperplasia, abnormal surface epithelium and increased infiltration of the lamina propria and intraepithelial compartments with inflammatory cells (8). Currently, the only way to normalize the typical clinical manifestations of the disease is to adhere to a life-long gluten-free diet, although some patients continue having gastrointestinal symptoms and tissue lesions and, especially, in refractory CD the activation of intraepithelial lymphocytes is not controllable with this diet and this situation can turn into T cell lymphoma (3).

Dendritic cells (DCs) are professional antigen presenting cells (APCs), which play a key role in intestinal

immune homeostasis, maintaining tolerance towards commensal microbes and generating appropriate immune responses against pathogens and antigens (9). Intestinal DCs are found in close association with epithelial cells and concentrated at sites of antigen entry (10). There are several possible routes by which DCs pick up antigens. One is after the transport of antigens to the lamina propria by transcytosis through M cells present in the follicle-associated epithelium of the Peyer's Patches. Another is by direct sampling of the antigens from the intestinal lumen by DCs, which can form TJ-like structures with intestinal epithelial cells and project dendrites through the epithelial cell layer and into the lumen (10,11). Untreated celiac disease mucosa harbors elevated numbers of activated DCs (12) and has recently been reported that those DCs are in fact newly recruited monocytes expressing CD14+CD11c+ whose accumulation is specific to CD and precedes changes in mucosal architecture (13). Moreover, *in vitro* studies reported that gliadins are able to induce phenotypic and functional maturation of DCs (14) as well as migration (15).

There have also been recent suggestions of the potential role of bacteria in the pathogenesis of CD as patients have an unbalanced gut microbiota composition in comparison with controls (16-19). Our previous research has shown that

immune responses of monocytes to gliadins and IFN- $\gamma$  can be regulated by specific intestinal bacteria, which could contribute to the Th1-type cytokine profile characteristic of the disease differently (20). Moreover, our studies report that different intestinal bacteria distinctly affect the permeability of the intestinal mucosal layer and the architecture of the epithelium layer in rat intestinal loops exposed to gliadin and IFN- $\gamma$  (21).

The aim of this study was to investigate the interactions of monocyte-derived DCs (MDDCs) with different intestinal bacteria (commensal and potential pathogens) in combination, or not, with gliadins, and co-cultured, or not, with Caco-2 cells. The effect on MDDCs morphology, adhesion and spreading, cytokine production and cell surface marker expression were determined. The ultimate purpose of our research is to gain a better understanding of the possible contribution of different intestinal bacteria to CD, based on their effects on the immune response to the main celiac disease trigger (gliadin).

## MATERIALS AND METHODS

### Gliadin fragments

Peptic fragments of gliadin (Sigma, St Louis, MO) were prepared using pepsin agarose gel (ICN, Biomedicals, Ohio) as previously described (22). Protein concentrations were

measured by Bicinchoninic acid assay (BCA Protein assay, Pierce, Rockford, IL).

### **Bacterial strains and culture conditions**

The following strains were used in the study: *B. longum* CECT 7347 (IATA-ES1), *B. bifidum* CECT 7365 (IATA-ES2), *Shigella* CBD8 and *E. coli* CBL2. *B. longum* CECT 7347 and *B. bifidum* CECT 7365 were previously isolated from healthy human feces and identified, as previously described (23-24). *E. coli* CBL2 was isolated from symptom-free CD patients and *Shigella* CBD8 was isolated from untreated CD patients, as previously described (25). Bifidobacteria were grown routinely in de Man, Rogosa and Sharpe (MRS) broth (Scharlau Chemie SA, Barcelona, Spain) with 0.05% cysteine (MRS-C) and incubated at 37°C under anaerobic conditions (AnaeroGen; Oxoid, Basingstoke, UK) for 22 h. Enterobacteria were routinely grown in Violet Red Bile Dextrose (VRBD) agar or Brain Heart broth (Scharlau Chemie SA, Barcelona, Spain) at 37°C for 24 h under aerobic conditions.

Cells were harvested by centrifugation (6,000 *g* for 15 min) at stationary growth phase, washed twice in phosphate buffered saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate, pH 7.4), and re-suspended in PBS containing 20% glycerol. Aliquots of these suspensions were frozen in liquid

nitrogen and stored at -80°C until used. The number of live cells after storage was determined by colony-forming unit (CFU) counting on MRS-C or VRBD agar after 48 h incubation in optimal conditions. For all strains tested, more than 90% cells were alive upon thawing and no significant differences were found during storage time (4 months). One fresh aliquot was thawed for every new experiment to avoid variability in CFU/ml between experiments.

### **Monocyte-derived DC (MDDC) isolation and stimulation**

MDDCs were generated as previously described (14). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors (provided by the Department of Blood Transfusion at Thomayer's Hospital, Prague, Czech Republic) by centrifugation over a Ficoll density gradient (Amersham Biosciences, Piscataway, NJ) and adjusted to  $3 \times 10^6$  cells/ml in 75-cm<sup>2</sup> plastic culture flasks (Corning, Costar, Madrid, Spain) in RPMI 1640 medium (BioWhittaker, Lonza, Belgium), supplemented with 10 % fetal bovine serum (FBS) (BioWhittaker, Lonza, Belgium), 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin (Sigma, Madrid, Spain). After 2 h, the non-adherent fraction of cells was washed away thoroughly and the isolated adherent monocytes were incubated with 500 U/ml GM-CSF (Immunotools,



Friesoythe, Germany) and 20 ng/ml recombinant human IL-4 (Immunotools, Friesoythe, Germany) in RPMI 1640 medium (BioWhittaker, Lonza, Belgium), supplemented with 10 % fetal bovine serum (FBS) (BioWhittaker, Lonza, Belgium), 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin (Sigma, Madrid, Spain) at 37° C under 5% CO<sub>2</sub>. After 5-6 days, the generated MDDCs were harvested and seeded at 1 x 10<sup>6</sup> cells/ml in 24-well flat-bottom polystyrene microtiter plates (Corning, Costar, Madrid, Spain). Then, cells were incubated for 24 hours with the following stimuli: gliadin (100 µg/ml) or bacterial cell suspensions (10<sup>6</sup> CFU/ml) alone or in combination with gliadin. Purified lipopolysaccharide (LPS) from *Salmonella enterica* subsp. *enterica* serotype Typhimurium (Sigma, St. Louis, MO) was used at a concentration of 1 µg/ml as a positive control. Non-stimulated PBMCs were also evaluated as controls of basal cytokine production. All reagents were tested by the E-toxate test for LPS (Sigma) and shown to be below the limit of detection (2 pg/ml). Cells were used for the experiments and cell culture supernatants were collected by centrifugation, fractionated in aliquots, and stored at -20°C until cytokines were analyzed. Every stimulus was assayed in duplicate in six different experiments.

### **Determination of cell adhesion and spreading**

Impedance measurements using xCelligence system in E-plates (Roche, Mannheim, Germany) were used to determine MDDCs adhesion and spreading. E-plates were coated with fibronectin (20 µg/ml, Roche) in PBS for 1 h at room temperature, washed with PBS and background was determined in 80 µl of medium using Real-Time Cell Analyzer (RTCA) station. Subsequently, 100 l of the MDDC suspension (1x10<sup>5</sup> cells/well), and 20 µl of stimulus (20 µg of gliadin, 10<sup>5</sup> CFU bacterial cells, 0.2 µg of LPS, 5 µg of poly I:C or a cocktail of cytokines) were added. Cells in E-plates were placed in the RTCA station for 24 h cultivation at 37°C in a 5% CO<sub>2</sub> atmosphere. Impedance was measured every 5 min for an initial 5 h period of cultivation, and every 15 min for the remaining period of 19 h. Impedance is a measure of the relative change in the electrical impedance at a certain frequency ( $f_n$ ) and was represented by the cell index (CI) values  $((R_i - R_0) / 15 \text{ (Ohm)}) / 15 \text{ (Ohm)}$ ;  $R_0$ : background resistance,  $R_i$ : individual time point resistance). Increase in cell spreading and adhesiveness leads to an increase in impedance, as cells attached on the electrode sensor surfaces act as insulators and, vice versa, cell detachment or rounding up leads to decrease in impedance. All experiments were performed in triplicate.

### **Fluorescence microscopy of the actin cytoskeleton**

For fluorescence microscopy of the DC cytoskeleton, coverslips were coated with fibronectin (20 µg/ml, Roche) in PBS for 1 h at room temperature. Then,  $2 \times 10^5$  MDDCs were seeded on fibronectin-coated coverslips and treated with the desired agents for 24 h at 37°C in a 5% CO<sub>2</sub> atmosphere before being fixed with 4% paraformaldehyde in PBS (20 min, RT). Cells were permeabilized with 0.1 % Triton X-100 in PBS (5 min), and incubated with TRITC-conjugated phalloidin (0.5 µg/ml, Sigma) in 2% BSA-PBS for 30 min to detect F-actin. Images were taken using an Olympus CellR fluorescence microscope.

### **Flow cytometry analysis of cell-surface markers**

MDDCs were stained with the following monoclonal antibodies at optimal concentrations: CD11c-allophycocyanin, CD14-APC-alexa fluor 750, CD83-PE (CALTAG), CD86-PE (Immunotech), CD40-FITC and HLA-DR-FITC (Pharmingen). MDDCs from migration assays were stained for surface expression of CCR7 with CCR7-PE Ab (R&D System, Minneapolis). Cells were also incubated with irrelevant antibodies at the same concentration and isotype. After two washing steps, the cells were resuspended in ice-cold PBS with 0.1% NaN<sub>3</sub> or fixed with 2% paraformaldehyde, and

surface markers were analyzed by flow cytometry (BD FACS Vantage SE, San Jose, California). Staining with Hoechst (Invitrogen, Gibco) was performed to assess cell viability. Data were analyzed using CellQuest software (BD Bioscience).

### **Enzyme linked immunosorbent assay (ELISA)**

Cytokine concentrations of cell supernatants were measured by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. The cytokines analyzed were IL-10, IL-12, TNF-α, IFN-γ (DuoSet Kit, R&D System, Minneapolis), IL-15 (eBioscience, San Diego, CA, USA) and IL-21 (Biolegend, San Diego, CA, USA).

### **Gene expression by Reverse-Transcription (RT) PCR**

The mRNA expression of the TJ proteins ZO-1 and occludin was analyzed by RT-PCR in Caco-2 and in MDDCs cultured alone or with Caco-2 cells as described below. Total RNA extraction was conducted with RNeasy mini kit (Qiagen, Madrid, Spain) according to the manufacturer's instructions. Total RNA was reversely transcribed to cDNA with ImProm-II Reverse Transcription System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. cDNA was then amplified with LightCycler® 480 SYBR Green I Master mix (Roche,

Barcelona, Spain) in a LightCycler® 480 Real-Time PCR System (Roche, Barcelona, Spain) using the primers shown in **Table 1**. The primers for occludin were designed using the Pubmed Primer Blast designing tool and the corresponding accession number is NM 002538.2. Data were normalized referring to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and analyzed with the  $\Delta\Delta C_t$  method.

### Caco-2 cell culture and co-cultures

The human enterocyte-like cell line Caco-2 was obtained from European Cell Culture collection (ECACC, 86010202, Salisbury, UK) and cultured in Dulbecco's Modified Eagle Medium (DMEM) Glutamax, containing 10% of inactivated (56° C for 30 min) fetal bovine serum (FBS) (Gibco, Barcelona, Spain), 100 µg/ml streptomycin and 100 U/ml penicillin (Sigma), 1% concentrated nonessential amino acid solution (Sigma) and 1% HEPES solution (Lonza, Madrid, Spain). For co-cultures,  $3.5 \times 10^5$  Caco-2 cells were seeded on the apical compartment

of 6.5 mm Transwell® polycarbonate inserts (24 wells, 3 µm membrane pore size) (Corning, Costar, Madrid, Spain) and cultivated for  $10 \pm 2$  days. During cell growth and differentiation, the medium in both compartments was replaced three times a week. Confluence was controlled by measurement of transepithelial electrical resistance (TEER; Millicell ERS Ohmmeter, Millipore, Madrid, Spain) and by visually detecting cell layer integrity under the microscope. Co-culture experiments of differentiated Caco-2 cells and MDDCs were performed in duplicate in five independent experiments. On day 10-12, MDDCs were added ( $1 \times 10^6$  cells per well) in the basolateral compartment of the transwell, while Caco-2 cells were previously cultured in the apical compartment. The stimuli were added to the apical compartment representing the intestinal epithelium. Controls of basal cytokine production by non-stimulated cells were run simultaneously in each assay. The following stimuli were added:

**Table 1.** Primers used in the study to analyze gene expression by RT-PCR.

Target gene	Sequence (5'-3')	References
ZO-1	ATCCCAAATAAGAACAGAGC GGCGTTACATCTAATAAAGC	(11)
Occludin	GGTGTGGGAAGCAGGACCGC GGAGAGGTGGACGCGGGAGT	This study
GAPDH	CCACCCATGGCAAATTCATGGCA TCTAGACGGCAGGTCAGGTCCACC	(27)

gliadin (100 µg/ml), bacterial cell suspension (10<sup>6</sup> CFU/ml) or bacterial cell suspension (10<sup>6</sup> CFU/ml) plus gliadin (100 µg/ml). Purified lipopolysaccharide (LPS) from *Salmonella enterica* subsp. *enterica* serotype Typhimurium (Sigma, St. Louis, MO) was used at a concentration of 1 µg/ml as positive control. Supernatants were collected after 24 hours and were stored at -20° C until cytokines were analyzed. Cells were also recovered and immediately kept at -80°C for further RNA extraction and gene expression analyses.

### Statistical analyses

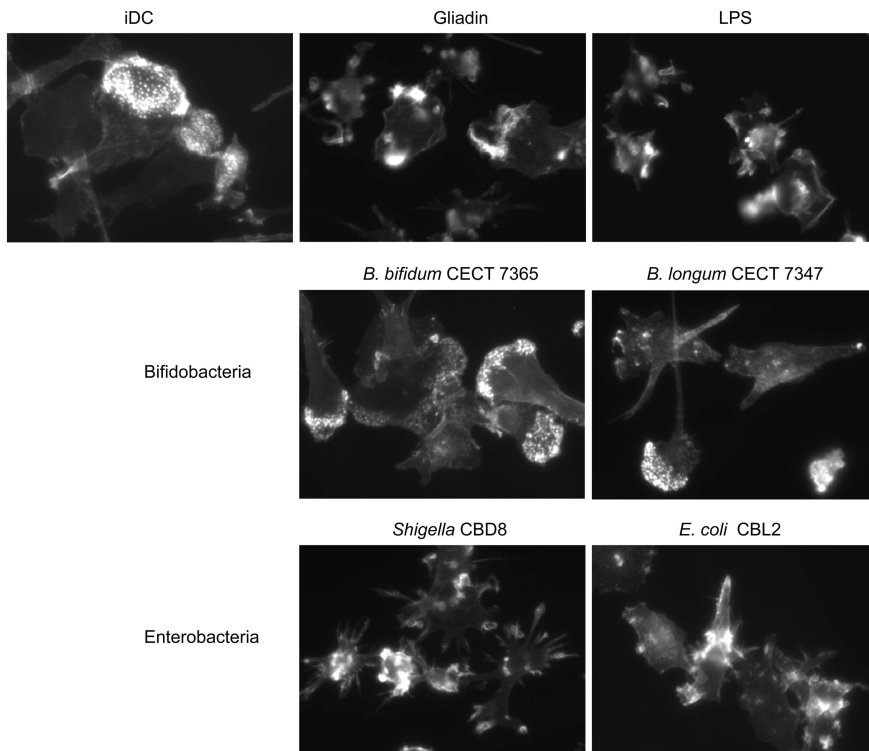
Statistical analyses were performed using SPSS version 17.0 software (SPSS Inc, Chicago, IL, USA). The Levene test was run in order to establish the homogeneity of variances and data distribution. Data distribution was not normal and variances were not homogeneous; therefore, Mann-Whitney U test was used to assess the effect of each variable. Data are expressed as medians and quartiles. Significant differences were established at a *P* value less than 0.05.

## RESULTS

### MDDC morphologic changes after interaction with intestinal bacteria

Actin cytoskeleton is known to be crucially involved in cell adhesion and spreading, which in

turn influence DC activation and maturation. Therefore, we evaluated the possible rearrangements of the actin cytoskeleton of MDDCs after stimulation with different intestinal bacteria (commensal and pathogens). As previously reported and shown in **Figure 1**, non-stimulated immature MDDCs exhibited the classical podosome structures, characterized by the actin-dense core which is surrounded by ring of cytoskeletal proteins (28, 29). On the contrary, gliadin fragments caused podosome dissolution and cytoskeletal remodeling as shown in **Figure 1**. Interestingly, MDDCs stimulated with *B. bifidum* CECT 7365 for 24 h were morphologically similar to non-stimulated cells. In a cell fraction stimulated with *B. longum* CECT 7347 for 24 h, podosome dissolution was observed, although a substantial portion of *B. longum*-stimulated cells still exhibited morphology similar to unstimulated cells or cells stimulated with *B. bifidum* CECT 7365. In contrast, MDDCs stimulated with *Shigella* CBD8 and *E. coli* CBL2 presented complete loss of podosomes and a strong amount of actin-rich protrusions, as observed for LPS-treated cells (**Figure 1**). MDDCs treated with LPS (1 µg/ml) for 24 h also displayed an evident induction of actin-rich protrusions (dendrites) and complete podosome dissolution. However, the enterobacteria-treated cells appeared more spread with irregular shapes, comparing to more cell-rounded morphology induced



**Figure 1.** Morphology and cytoskeletal remodeling in MDDCs plated on fibronectin-coated coverslips and under the effect of different stimuli: Immature MDDCs (iDC), gliadin (100  $\mu\text{g}/\text{ml}$ ), LPS (1  $\mu\text{g}/\text{ml}$ ), *B. bifidum* CECT 7365 ( $10^6$  CFU), *B. longum* CECT 7347 ( $10^6$  CFU), *Shigella* CBD8 ( $10^6$  CFU) and *E. coli* CBL2 ( $10^6$  CFU) for 24 h.

by LPS. The observed changes seem to be in line with those observed with the dynamic monitoring of cell adhesion and spreading described below. The enterobacteria seemed to induce cytoskeletal remodeling and mediate the morphological transition from immature DC to mature DC, whereas activation of MDDCs by bifidobacteria was weaker.

### **MDDC adhesion and spreading influenced by intestinal bacteria**

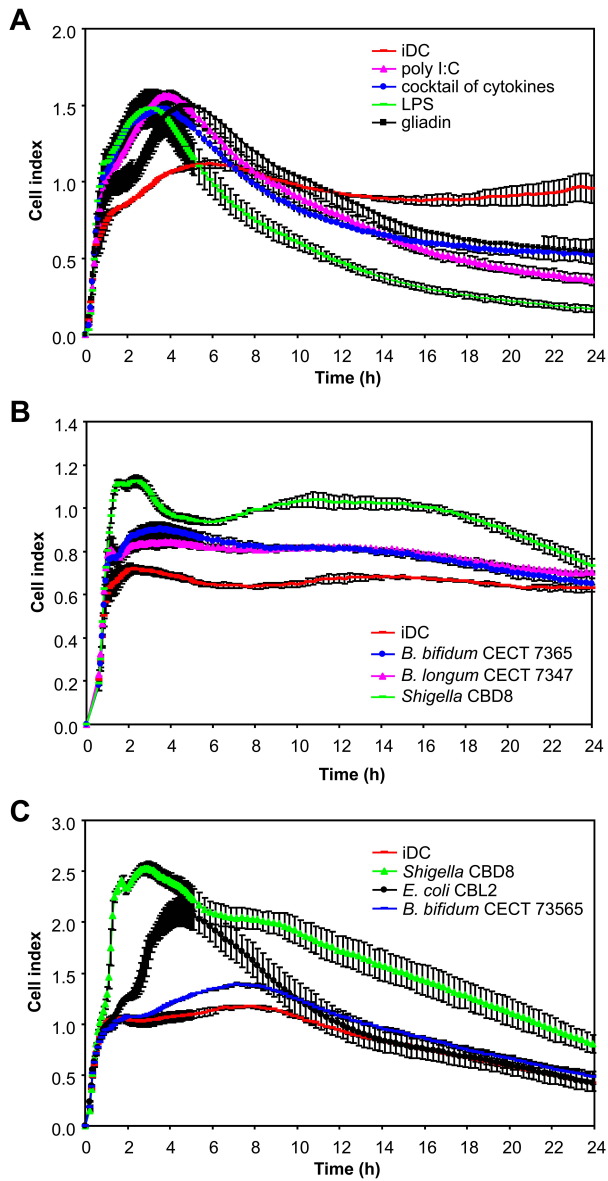
DC activation and maturation affects cell adhesion and spreading (30, 31). Therefore, to characterize the immunoregulatory potential of different intestinal bacteria (commensal and potential pathogens), we further sought to non-invasively determine their impact on MDDC adhesion and

spreading. To achieve this aim, impedance measurements were employed using the xCelligence system in fibronectin-coated E-plates. Indeed, the interaction of cells with fibronectin-coated microelectrodes in the E-plate generates an impedance change reflected in the CI that is proportional to the number of adhered cells and overall morphology and adhesion quality of the cells (31). The stimulation of DCs with maturation-inducing agents (e.g. a cocktail of pro-inflammatory cytokines, LPS, gliadins or poly I:C) resulted in a rapid increase in CI values corresponding to elevated cell adhesion and spreading when compared to non-stimulated cells. Within 24 h, however, the CI of stimulated DC dropped below the CI of control cells due to progressing maturation and cell rounding up (representative example shown in **Figure 2A**). It should be noted that the absolute CI values differ among individual donors (e.g. for iDC ranges from 0.5 to 1.2). However, the pattern of induced changes in response to tested components was very similar across various donors (see representative experiments Fig. 2, B and C). Importantly, addition of bifidobacteria (*B. longum* CECT 7347 and *B. bifidum* CECT 7365) led to a slight increase in cell adhesion (**Figure 2B**), while exposure to enterobacteria (*E. coli* CBL2 and *Shigella* CBD8) yielded greater activation of cell adhesion and spreading (**Figure 2C**). Although the

CI curves of enterobacteria exhibited a maximum which was followed by CI decrease, they did not generally drop below the CI of control cells within 24 h (6 experiments out of 9). Overall, the results indicate stronger activation of MDDCs by enterobacteria than by bifidobacteria consistently with data demonstrated in **Figure 1**. When gliadins were added to bifidobacteria, a slight increase in MDDC adhesion and spreading was observed, compared to stimulation with bifidobacteria alone. In contrast, the addition of gliadins to enterobacteria did not significantly change their response curves (**data not shown**).

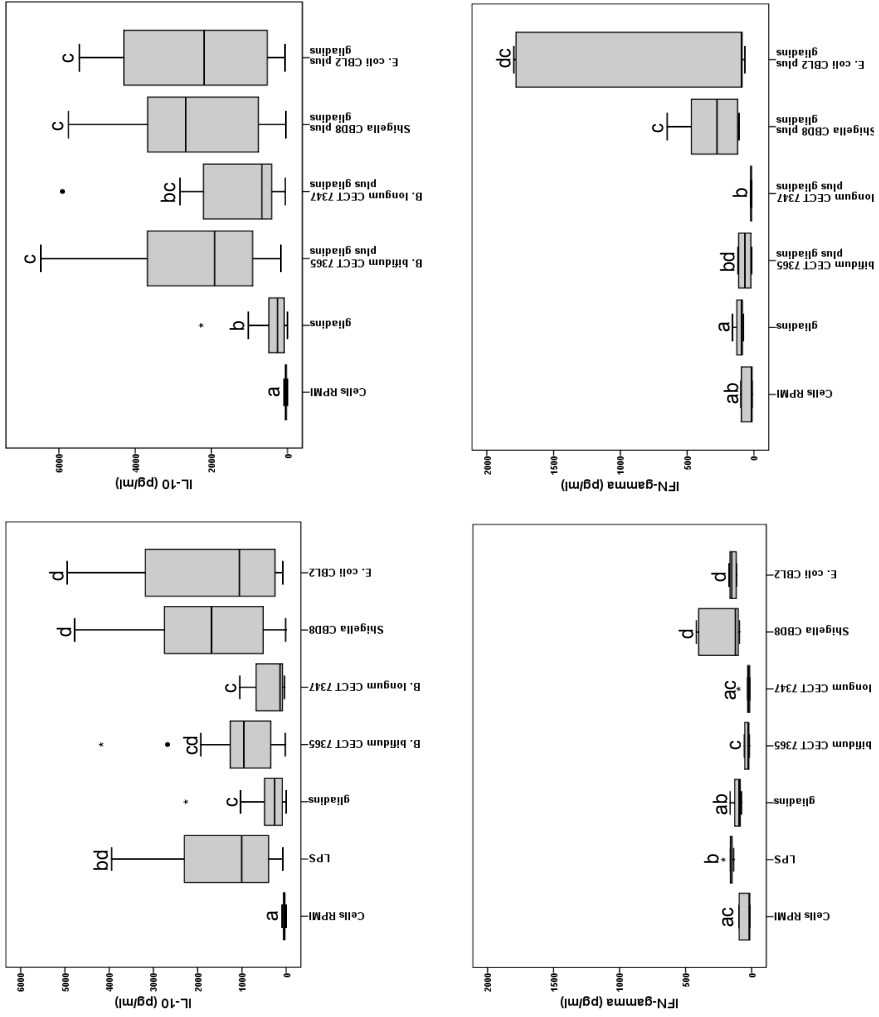
### **MDDCs cytokine production in response to intestinal bacteria and gliadin**

Cytokine production by MDDCs after stimulation with cell suspensions of different bacterial strains is shown in **Figure 3**. All the stimuli applied induced a higher production of IL-10, IL-12 and TNF- $\alpha$  ( $P \leq 0.001-0.001$ ) in comparison with control cells. However, only Gram-negative bacteria (*E. coli* CBL2 and *Shigella* CBD8) and LPS induced a greater secretion of IFN- $\gamma$  than non-stimulated cells ( $P \leq 0.001-0.028$ ). Gliadin also increased IFN- $\gamma$  secretion to a greater extent than *B. bifidum* CECT 7365 ( $P = 0.025$ ). When comparing different bacterial strains, the Gram-negative bacteria *Shigella* CBD8 induced a greater secretion of IL-12, TNF- $\alpha$  and



**Figure 2.** Representative data are shown for the different attachment and spreading of MDDCs (from 3 different donors) under different stimuli, in a 24 h time-course experiment. The attachment and spreading of MDDCs is expressed as CI/Time (see Materials and Methods). iDCs from donor A were exposed to gliadin, LPS; polyI:C and cocktail of cytokines; from donor B to bifidobacteria and from C to enterobacteria; *Shigella* CBD8 was used with B and C cells for comparison.

**Figure 3.** MDDCs cytokine production (IL-10, TNF- $\alpha$ , IFN- $\gamma$  and IL-12) after 24 h stimulation with gliadins (100  $\mu$ g/ml), bacterial cell suspensions ( $10^6$  CFU/ml) alone or in combination with gliadin. Statistically significant differences were outlined with different letters. A  $P < 0.05$  was considered statistically significant. (continued on next page)





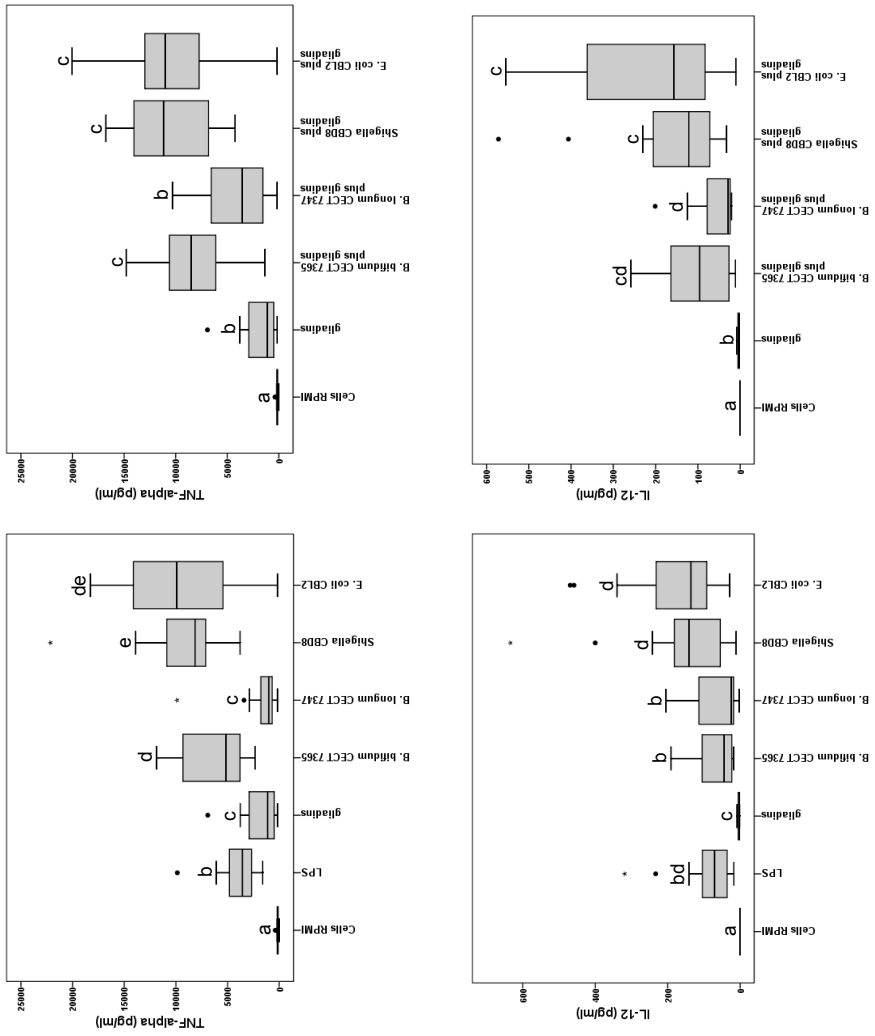


Figure 3. (continued)

IFN- $\gamma$  than the Gram-positives *B. bifidum* CECT 7365 and *B. longum* CECT 7347 ( $P= 0.028$  and  $P= 0.025$ ,  $P= 0.028$  and  $P<0.001$ ,  $P= 0.009$  and  $P= 0.016$ , respectively). Similarly, the Gram-negative bacteria *E. coli* CBL2 induced a greater secretion of IL-12 and IFN- $\gamma$  than *B. bifidum* CECT 7365 ( $P=0.004$  and  $P=0.006$ , respectively) and *B. longum* CECT 7347 ( $P=0.004$  and  $P=0.006$ , respectively). Moreover, *E. coli* CBL2 induced a greater secretion of TNF- $\alpha$  than *B. longum* CECT 7347 ( $P= 0.003$ ). *Shigella* CBD8 and *E. coli* CBL2 also induced a greater secretion of IL-10 than *B. longum* CECT 7347 ( $P= 0.018$ ). *B. longum* CECT 7347 induced lower production of TNF- $\alpha$  than *B. bifidum* CECT 7365 ( $P<0.001$ ). IL-21 secretion was extremely low and no differences were detected between the different stimuli applied (**data not shown**). IL-15 was not detected in supernatants of stimulated MDDCs.

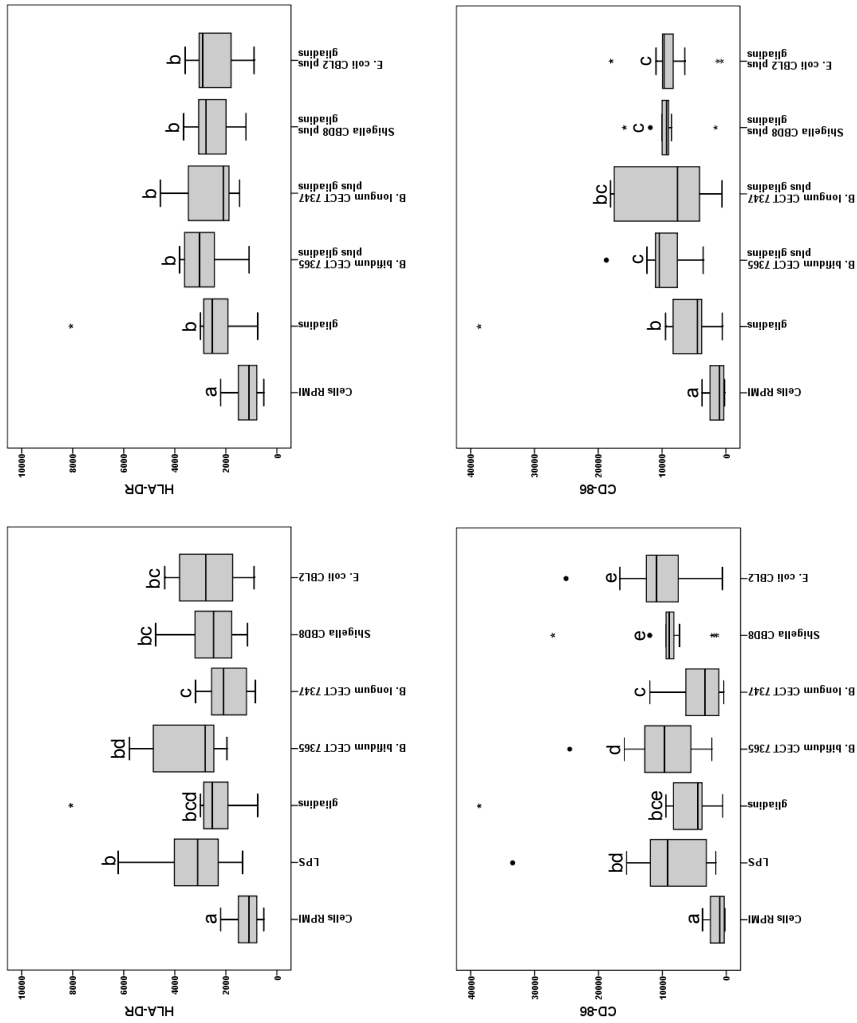
Cytokine production by MDDCs after stimulation with cell suspensions of different bacterial strains combined with gliadins is also shown in **Figure 3**. The effects of stimulating MDDCs with bacterial strains plus gliadins on cytokine production were similar to those exerted by the bacterial strains alone with some exceptions; the Gram-positive *B. longum* CECT 7347 plus gliadins reduced the IFN- $\gamma$  production induced by gliadins alone ( $P=0.010$ ). Furthermore, *B. longum* CECT 7347 induced higher

secretion of IL-10 when combined with gliadin than when used as sole stimulus ( $P= 0.045$ ). No differences were detected for the other strains.

### **MDDCs surface molecule expression in response to intestinal bacteria and gliadin**

MDDCs surface molecule expression is shown in **Figure 4**. Expression of all surface molecules, HLA-DR, CD-86, CD-40 and CD-83, was up-regulated by all stimuli applied when compared with non-stimulated cells ( $P<0.001-0.009$ ). The Gram-negative bacteria *Shigella* CBD8 and *E. coli* CBL2 induced higher CD-86 and CD-40 expression than *B. longum* CECT 7347 ( $P=0.002-0.007$ ). *B. bifidum* CECT 7365 induced higher HLA-DR, CD-86, CD-40 and CD-83 expression than *B. longum* CECT 7347 ( $P=0.002-0.009$ ). All stimuli combined with gliadins upregulated the expression of all surface markers in comparison with non-stimulated cells and the effects were similar to those exerted by the bacterial strains alone. *B. bifidum* CECT 7365 and *Shigella* CBD8 plus gliadins induced higher CD-86 and CD-83 expression than gliadins alone ( $P=0.008-0.046$ ). Likewise, *E. coli* CBL2 plus gliadins also induced higher CD-86 than gliadins alone ( $P=0.033$ ).

**Figure 4.** MDDCs surface marker expression after 24 h stimulation with gliadins (100 µg/ml), bacterial cell suspensions (10<sup>6</sup> CFU/ml) alone or in combination with gliadin. Statistically significant differences were outlined with different letters. A  $P < 0.05$  was considered statistically significant. (continued on next page)



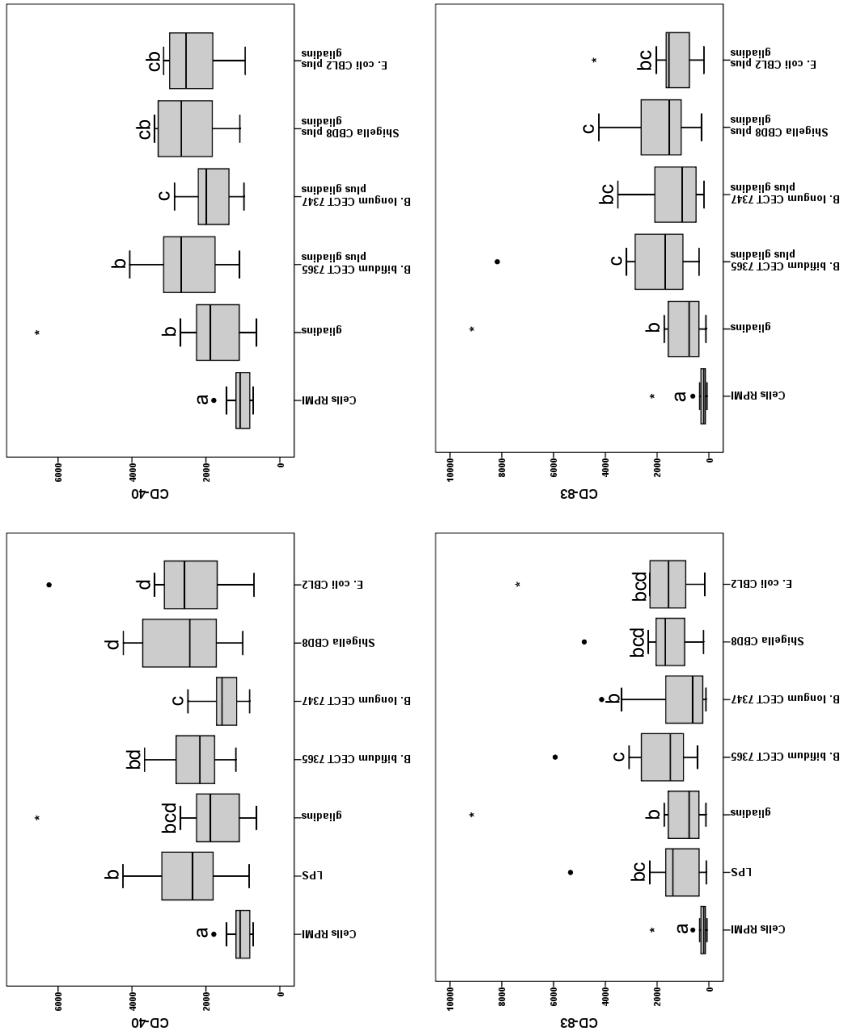


Figure 4. (continued)

### **Cytokine production and surface molecule expression by MDDCs co-cultured with Caco-2 cells stimulated with intestinal bacteria and gliadin**

Cytokine production by MDDCs in co-culture with Caco-2 cells, after stimulation of Caco-2 cells with bacterial cell suspensions was evaluated (**Supplementary Table 1**). The Gram-negative bacteria *Shigella* CBD8 and *E. coli* CBL2 induced higher IFN- $\gamma$  and TNF- $\alpha$  secretion by MDDCs than *B. bifidum* CECT 7365 ( $P=0.010-0.035$ ). *Shigella* CBD8 also induced higher IFN- $\gamma$  secretion ( $P=0.010$ ) and *E. coli* CBL2 higher TNF- $\alpha$  secretion ( $P=0.088$ ) by MDDCs than *B. longum* CECT 7347 although in the last case statistical significance was not reached.

Cytokine production by MDDCs in co-culture with Caco-2 cells, after stimulation of Caco-2 cells with bacterial cell suspensions combined with gliadins, was also analyzed, but no differences were detected compared to cytokine production induced by each bacterial strain alone (**Supplementary Table 2**). No statistically significant differences were detected for IL-21, IL-15 and IL-12 production possibly due to the low concentrations detected.

For surface marker expression in MDDCs co-cultured with Caco-2 cells, significant effects were only detected when *Shigella* CBD8 was used to stimulate Caco-2 cells, which increased CD-83 expression

by MDDCs compared to non-stimulated cells ( $P=0.029$ ), gliadins ( $P=0.029$ ) and *B. longum* CECT 7347 ( $P=0.029$ ). No differences were detected among different stimuli or between each stimulus alone or the stimulus plus gliadins (**data not shown**).

### **TJ protein mRNA expression in cultures of Caco-2 cells and MDDC analyzed by RT-PCR**

The effects of intestinal bacteria and gliadins on TJ protein expression in a differentiated monolayer of Caco-2 cells were analyzed after 24 h of stimulation. In these conditions, the expression of ZO-1 and occludin genes differed significantly among different stimuli (**Table 2**). *B. longum* CECT 7347 alone significantly up-regulated ZO-1 expression compared with non-stimulated cells ( $P=0.005$ ), LPS ( $P=0.009$ ) and gliadins ( $P=0.009$ ), whereas LPS and gliadins significantly down-regulated ZO-1 expression compared to controls ( $P=0.005$ ). Moreover, *B. longum* CECT 7347 up-regulated ZO-1 expression in comparison with *B. bifidum* CECT 7365 ( $P=0.028$ ). *B. longum* CECT 7347 plus gliadins also increased ZO-1 expression in comparison to gliadins alone ( $P=0.009$ ) and *B. longum* CECT 7347 alone ( $P=0.047$ ). Occludin expression followed a slightly different pattern, since gliadins did not significantly affect the expression. However, the other stimuli, including *B. bifidum* CECT

**Table 2:** ZO-1 and occludin mRNA expression in differentiated monolayer of Caco-2 cells after 24 h stimulation analyzed by RT-PCR.

Stimuli	ZO-1	(Min-Max)	Occludin	(Min-Max)
Non-stimulated cells (control)	1.00	(1.0-1.0)	1.00	(1.0-1.0)
<i>B. bifidum</i> CECT 7365	0.99	(0.40-1.14)	0.63	(0.5-0.8)
<i>B. bifidum</i> CECT 7365 plus gliadins	0.81	(0.17-0.95)	0.39	(0.38-0.40)
<i>B. longum</i> CECT 7347	1.18	(1.03-2.57)	0.66	(0.64-0.69)
<i>B. longum</i> CECT 7347 plus gliadins	1.68	(1.48-3.81)	0.89	(0.70-1.09)
<i>E. coli</i> CBL2	1.01	(0.28-1.27)	0.56	(0.44-0.71)
<i>E. coli</i> CBL2 plus gliadins	1.70	(0.17-1.79)	0.57	(0.41-0.80)
<i>Shigella</i> CBD8	1.81	(0.76-1.98)	0.91	(0.89-0.92)
<i>Shigella</i> CBD8 plus gliadins	1.36	(0.46-1.46)	0.86	(0.84-0.89)
LPS	0.76	(0.12-0.89)	1.35	(1.29-1.42)
Gliadins	0.84	(0.09-0.99)	1.02	(0.98-1.06)

Data are expressed as median with maximum and minimum of  $\Delta\Delta C_t$  values.

7365, *E. coli* CBL2, *Shigella* CBD8 and *B. longum* CECT 7347, slightly down-regulated occludin expression ( $P=0.014-21$ ) with the exception of LPS, which up-regulated it ( $P=0.014$ ). On comparing different bacterial stimuli, *Shigella* CBD8 decreased occludin gene expression to a lower extent than *B. bifidum* CECT 7365, *B. longum* CECT 7347 and *E. coli* CBL2 ( $P=0.021$ ). When combined with gliadins, *B. bifidum* CECT 7365 and *Shigella* CBD8 decreased occludin expression to a significantly greater extent than the corresponding strains alone ( $P=0.021$ ), whereas *B. longum* CECT 7347 significantly increased it compared to the strain alone ( $P=0.021$ ).

When gene expression of TJ proteins was analyzed in MDCCs co-cultured with Caco-2 cells, differences were

detected for ZO-1 and occludin expression among different stimuli (**Table 3**), but not when MDCCs were cultured alone. In MDCCs co-cultured with Caco-2 cells, ZO-1 gene expression was down-regulated by LPS ( $P<0.001$ ) and gliadins ( $P=0.017$ ) in comparison with non-stimulated cells, while the effects of the bacterial strain tested were not significant due to their large variability. When the bacterial cell suspensions were combined with gliadins, differences were detected for *Shigella* CBD8 plus gliadins, which decreased ZO-1 expression in MDCCs as compared with *Shigella* CBD8 alone ( $P=0.043$ ) or non-stimulated cells ( $P<0.001$ ) or *B. bifidum* CECT 7365 ( $P=0.010$ ). Furthermore, *B. bifidum* CECT 7365 plus gliadins upregulated ZO-1 expression compared to gliadins alone

**Table 3:** ZO-1 and occludin mRNA expression in MDDCs co-cultured with stimulated Caco-2 cells analyzed by RT-PCR.

Stimuli	ZO-1	(Min-Max)	Occludin	(Min-Max)
Non-stimulated cells (control)	1.00	(1.0-1.0)	1.00	(1.0-1.0)
<i>B. bifidum</i> CECT 7365	1.41	(0.15-3.45)	1.26	(0.91-1.42)
<i>B. bifidum</i> CECT 7365 plus gliadins	4.43	(0.53-11.08)	1.20	(1.20-1.21)
<i>B. longum</i> CECT 7347	0.72	(0.16-5.54)	0.77	(0.42-1.16)
<i>B. longum</i> CECT 7347 plus gliadins	3.26	(0.21-7.81)	2.20	(1.91-2.57)
<i>E. coli</i> CBL2	1.20	(0.22-4.57)	1.07	(1.04-1.11)
<i>E. coli</i> CBL2 plus gliadins	1.51	(0.43-3.27)	1.68	(1.58-1.77)
<i>Shigella</i> CBD8	0.82	(0.20-2.25)	1.28	(1.11-1.36)
<i>Shigella</i> CBD8 plus gliadins	0.48	(0.11-0.81)	1.02	(0.99-1.06)
LPS	0.0027	(0.0027-0.59)	2.47	(2.42-2.52)
Gliadins	0.58	(0.11-1.03)	1.18	(1.13-1.23)

Data are expressed as mean and standard deviation of  $\Delta\Delta C_t$  values.

( $P=0.050$ ). In relation to occludin gene expression in MDDCs co-cultured with Caco-2 cells, the effects of gliadin alone were not significant, however LPS enhanced occludin expression in comparison with cells alone ( $P=0.050$ ). Moreover, *B. longum* CECT 7347 plus gliadins enhanced occludin expression in comparison to *B. longum* CECT 7347 alone ( $P=0.010$ ).

## DISCUSSION

This is the first study to provide evidence for the differential influence of intestinal bacteria and their interactions with gliadins on MDDCs morphology and dynamic attachment and spreading. These data may reflect the phenotypic changes DCs undergo, upon stimulation, associated with their maturation

process and conversion from a tissue resident, antigen-capturing cell to a highly migratory antigen-presenting cell (32, 33). Extensive changes in morphology, cytoskeletal organization and cell adhesion are considered indexes of the maturation and transition process that may ultimately influence T-cell activation (33). In particular, podosomes are sites of active matrix degradation, which promote cell migration and invasion, and their presence is peculiar to immature DCs, whereas mature DCs are loosely adherent and not forming podosomes on fibronectin but characteristic actin-rich extensions (dendrites) that maximize the contact area with T-cells (33). In our study, *Shigella* CBD8, *E. coli* CBL2, LPS and gliadins induced major changes in MDDC morphology, which could reflect a mature phenotype, whereas *B.*

*bifidum* CECT 7365 and *B. longum* CECT 7347 did not induce evident morphological changes. These results are in agreement with those by Chladkova *et al.* (15) who described that gliadin is able to induce the loss of podosomes in a dose-dependent manner and that gliadin fragments induce cytoskeletal remodeling and mediate the morphological transition from immature DC to mature DC. These morphological data are in agreement with dynamic adhesion and spreading assays. When DCs are immature they are highly adhesive and possess podosomes, then on undergoing maturation they start to change their morphology, lose podosomes and acquire dendrites. We observed that *Shigella* CBD8, with and without gliadins, induced faster and more remarkable changes in adhesion and spreading in MDCCs than the other stimuli. *E. coli* CBL2 also induced changes while the bifidobacterial strains, particularly *B. bifidum* CECT 7365, had no significant effect on the cells and even reduced the effect of gliadins resembling the situation of non-stimulated cells (**data not shown**). The attachment and spreading experiments show the interaction of MDCCs with fibronectin, which composes ECM components and interacts specifically with the cells through specialized cell-surface receptors called integrins. The latter recognize and bind to specific motifs within the ECM proteins, thereby mediating the ability of cells to

specifically adhere to and interact with the appropriate matrix protein (31). Integrins also initiate an intracellular signaling cascade that directs cellular processes such as cell survival, proliferation, differentiation and migration (31, 34).

Overall, the differential effects of the intestinal bacteria tested on MDCCs morphology, adhesion and spreading suggest that enterobacteria, in particular, or their cell-wall components could contribute to inducing a full phenotypic and functional DC maturation process and thus enhance antigen presentation and stimulation of specific T-cell responses.

Subsequently, we also evaluated the effects of different intestinal bacteria on direct markers of MDCC immune function, such as cytokine production and surface molecule expression, as well as their ability to modify the response to gliadin. The two enterobacteria and, especially, *Shigella* CBD8 that represent a real pathogen triggered a stronger Th1-type inflammatory response, with particularly high IFN- $\gamma$ , TNF- $\alpha$  and IL-12 secretion compared to the effects of the two bifidobacterial strains tested. Similarly, *E. coli* CBL2 also induced greater IFN- $\gamma$  and IL-12 secretion than the two bifidobacterial strains tested. These effects on the cytokine profile followed a similar trend to those detected in PBMCs in our previous study (20), but were more pronounced. In addition, this study shows that gliadin stimulation



induced higher IFN- $\gamma$  secretion than *B. bifidum* CECT 7365, which was not evident in previous studies with PBMCs (20). Moreover, *B. longum* CECT 7347 plus gliadins reduced the levels of IFN- $\gamma$  production induced by gliadins alone and also induced higher secretion of IL-10 than when the bacterium was used as the sole stimulus. This would suggest its potential role in the regulation of the aberrant immunological response induced by wheat gluten proteins. In relation to MDDC surface molecule expression, the differences between enterobacteria and bifidobacteria were not as remarkable as those detected in PBMCs (20). However, *B. longum* CECT 7347 clearly induced lower expression of most of the activation markers when compared with *Shigella* CBD8, *E. coli* CBL2 and *B. bifidum* CECT 7365 in the presence or absence of gliadins.

Our study also examined the role of the epithelial cell layer in the response of MDDCs to different stimuli. Similar results were observed when MDDCs were co-cultured with differentiated Caco-2 cells and stimulated with gliadin and different bacterial strains. Unlike bifidobacteria, enterobacteria induced higher production of pro-inflammatory cytokines, with predominant increases in IFN- $\gamma$  and TNF- $\alpha$ , which could contribute to the intestinal inflammatory milieu characteristic of CD patients. Accordingly, stool samples of CD

patients, representing an unbalanced microbiota, were also shown to induce a pro-inflammatory cytokine pattern characterized by high levels of IFN- $\gamma$  and TNF- $\alpha$  production by PBMCs. However, this imbalance could be reversed by the two bifidobacterial strains tested in this study (35), suggesting that intestinal bacteria could influence the polarization of the immunological responses in the presence of factors influencing CD, including the luminal bacterial load and wheat-gluten-derived peptides.

The effects of different bacterial strains and gliadins on expression of TJ-related proteins in Caco-2 cells were also studied as CD has also been associated with increases in intestinal permeability and changes in expression of TJ related proteins (36, 37). In this study, gliadin and LPS were shown to reduce ZO-1 gene expression in Caco-2 cells, while *B. longum* CECT 7347 increased ZO-1 expression alone and in combination with gliadins, reversing this adverse effect. The effects of the different bacterial strains on occludin expression followed opposite trends, but their meaning in the context of CD is unclear since gliadins did not modify occluding expression. Previous *in vitro* studies, using monolayer Caco-2 cell cultures and three-dimensional Lovo cell line culture, indicated that gliadin directly damages TJ, for example decreasing expression of ZO-1 and occluding proteins, thereby contributing to gut barrier disruption

(37, 39). In patients with active coeliac disease, both ZO-1 protein levels and mRNA were reduced and F-actin organization was disrupted (40). Similarly, Drago *et al.* (38) reported significant lower expression of both ZO-1 and occluding genes in the intestinal tissues obtained from CD patients, compared to non-CD controls, which represent the effect of chronic exposure to gluten.

DCs expressing TJ proteins are reported to open the TJ between epithelial cells and directly take up microorganisms or other antigens, thus contributing to antigen translocation, while preserving integrity of the epithelial barrier (11, 41). Thus the effects of different bacterial strains and gliadins on the expression of TJ related proteins in MDDCs were also evaluated in our study. Differences in TJ protein gene expression on MDDCs among different stimuli were only detected when these cells were co-cultured with Caco-2 cells. These findings suggest that the interactions with Caco-2 cells play an important role in MDDC recognition of different stimuli, which may lead to different TJ protein expression during trans-epithelial exchanges. In this context, previous studies demonstrated that particle uptake by MDDCs was also higher in co-cultures with epithelial cells (41). LPS and gliadins reduced ZO-1 expression in agreement with the effects on Caco-2 cells. However, *B. bifidum* CECT 7365 plus gliadins upregulated ZO-1 expression

compared to gliadins alone. In relation to occludin, LPS increased the expression of this protein gene while gliadins and most of the bacteria did not exert significant effects. Only *B. longum* CECT 7347 combined with gliadin increased occludin expression compared with the strain alone. Bacterial components, such as LPS, are reported to trigger the reorganization of TJ proteins via up-regulation of ZO-1 and slight down-regulation of occludin, allowing DCs to detach from junctions with epithelial cells and migrate to draining lymph nodes (11). By contrast, in our study the effects of LPS on ZO-1 and occludin expression and those of the enterobacteria, which represent an LPS stimulus, on occludin expression followed the opposite trend. These differences could be explained by differences in mRNA expression of these genes at different stages of DCs maturation and exposure time as indicated previously (11). In our study, the effects of *B. longum* CECT 7347 on ZO-1 expression were opposite to those of LPS. This would suggest that this bifidobacterial strain could play a positive role in the regulation of TJ related protein expression; however, further investigations are required to provide direct evidence of the physiological significance of the effects detected in TJ protein gene expression.

In conclusion, this study confirms that enterobacteria could exert pathogenic effects similar to those

exerted by gliadins, contributing to phenotypic DC maturation. This is reflected in morphological changes and increased attachment/spreading ability, and the induction of high inflammatory cytokine production, particularly of IFN- $\gamma$ . In contrast, *B. bifidum* CECT 7365 and *B. longum* CECT 7347 did not cause gliadin-induced morphological changes, and induced adhesion and spreading ability of MDDCs and inflammatory cytokine production to a lesser extent. In particular, *B. longum* CECT 7347 reduced IFN- $\gamma$  production induced by gliadins and increased IL-10 secretion when both stimuli were combined and also reversed the gliadin-reduced ZO-1 expression in Caco-2 cells. Altogether this could ultimately contribute to ameliorating the response to gluten peptides in the context of CD. Based on these results, we suggest that specific components of the gut microbiota may influence phenotypic and functional maturation of DCs differently and their interactions with epithelial cells, and could ultimately define the role of DCs in T-cell activation and disease progression.

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**Sup. table 1:** Cytokine secretion (pg/ml) of MDDCs co-cultured with Caco-2 cells stimulated with bacterial suspensions (106/ml) or gliadins (100 µg/ml) or LPS (1 µg/ml).

Stimuli	IL-10	(Min-Max)	TNF-α	(Min-Max)	IFN-γ	(Min-Max)	IL-12	(Min-Max)
Non-stimulated cells (control)	95.05 <sup>a</sup>	(85.58-420.01)	32.74 <sup>ac</sup>	(2.25-52.16)	48.81 <sup>a</sup>	(0.17-85.92)	0.12 <sup>a</sup>	(0.07-0.16)
LPS	465.70 <sup>b</sup>	(444.92-486.48)	483.57 <sup>bc</sup>	(483.14-484.00)	94.75 <sup>b</sup>	(93.87-95.63)	0.07 <sup>ab</sup>	(0.07-0.07)
Gliadins	127.51 <sup>a</sup>	(71.56-399.40)	43.97 <sup>abcd</sup>	(1.12-115.39)	36.90 <sup>abd</sup>	(1.12-98.31)	0.16 <sup>ab</sup>	(0.16-0.16)
<i>B. bifidum</i> CECT 7365	157.83 <sup>ac</sup>	(77.65-411.90)	11.37 <sup>c</sup>	(0.17-28.89)	21.70 <sup>ab</sup>	(3.39-108.97)	0.12 <sup>ab</sup>	(0.07-0.16)
<i>B. longum</i> CECT 7347	313.22 <sup>cb</sup>	(159.99-558.0)	90.64 <sup>d</sup>	(47.25-240.40)	48.98 <sup>ad</sup>	(1.12-91.61)	3.23 <sup>b</sup>	(0.26-6.49)
<i>Shigella</i> CBD8	387.50 <sup>b</sup>	(164.06-616.83)	264.20 <sup>d</sup>	(43.28-836.09)	85.94 <sup>cb</sup>	(38.79-241.77)	1.01 <sup>ab</sup>	(0.07-2.43)
<i>E. coli</i> CBL2	202.34 <sup>cb</sup>	(98.41-633.32)	424.74 <sup>d</sup>	(92.59-901.03)	57.03 <sup>d</sup>	(27.71-107.02)	1.87 <sup>b</sup>	(0.47-4.07)

\* Different letters mean statistically significant differences established by applying the Mann-Whitney U test. A P<0.050 was considered statistically significant.

**Sup. table 2:** Cytokine secretion (pg/ml) of MDDCs co-cultured with Caco-2 cells stimulated with bacterial suspensions (106/ml) in combination with gliadins (100 µg/ml).

Stimuli	IL-10	(Min-Max)	TNF-α	(Min-Max)	IFN-γ	(Min-Max)	IL-12	(Min-Max)
Non-stimulated cells (control)	95.05 <sup>a</sup>	(85.58-420.01)	32.74 <sup>a</sup>	(2.25-52.16)	48.81 <sup>ab</sup>	(0.17-85.92)	0.12 <sup>a</sup>	(0.07-0.16)
Gliadins	127.51 <sup>a</sup>	(71.56-399.40)	43.97 <sup>a</sup>	(1.12-115.39)	36.90 <sup>ab</sup>	(1.12-98.31)	0.16 <sup>ab</sup>	(0.16-0.16)
<i>B. bifidum</i> CECT 7365 plus gliadins	99.68 <sup>ab</sup>	(83.48-434.34)	15.07 <sup>a</sup>	(1.66-19.12)	21.62 <sup>a</sup>	(1.66-86.86)	0.67 <sup>ab</sup>	(0.26-1.07)
<i>B. longum</i> CECT 7347 plus gliadins	313.22 <sup>bc</sup>	(195.36-440.56)	251.17 <sup>b</sup>	(178.82-645.43)	29.09 <sup>a</sup>	(3.30-98.31)	0.54 <sup>b</sup>	(0.16-1.20)
<i>Shigella</i> CBD8 plus gliadins	387.50 <sup>c</sup>	(197.13-451.13)	264.20 <sup>b</sup>	(186.34-443.63)	54.33 <sup>ab</sup>	(12.87-117.80)	0.90 <sup>ab</sup>	(0.07-1.60)
<i>E. coli</i> CBL2 plus gliadins	379.00 <sup>c</sup>	(193.77-496.37)	158.18 <sup>b</sup>	(66.82-658.04)	81.73 <sup>b</sup>	(47.35-98.31)	4.39 <sup>ab</sup>	(0.16-5.18)

\* Different letters mean statistically significant differences established by applying the Mann-Whitney U test. A P<0.050 was considered statistically significant.

# **Influence of *Bifidobacterium longum* CECT 7347 and gliadin peptides on intestinal epithelial cell proteome**

Marta Olivares, Moisés Laparra, and Yolanda Sanz

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## ABSTRACT

Celiac disease is an enteropathy caused by an abnormal immune response to cereal gluten proteins (gliadin). To unravel the possible role of the interactions between gliadin peptides and specific intestinal bacteria, the response of intestinal epithelial (Caco-2) cells to gliadin subjected to gastrointestinal digestion in the presence or absence of *Bifidobacterium longum* CECT 7347 has been studied. Changes in the proteome of Caco-2 cells were determined by 2DE and MALDI-TOF. Gliadins digested without *B. longum* altered the expression of a higher number of proteins than in the presence of the bacterium (21 versus 9) and these proteins were involved in disorganization of cell cytoskeleton, inflammation and apoptosis. Gliadins digested in the presence of the bacterium influenced the production of proteins involved in calcium homeostasis and cell survival and function. Therefore, *B. longum* CECT 7347 might ameliorate gliadin toxicity and modify the responses of intestinal epithelial cells to the gliadin challenge.

**Keywords:** *Bifidobacterium longum*, celiac disease, Caco-2 cells, probiotic, proteome

## INTRODUCTION

Cereal gluten proteins (gliadin  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\omega$ ) are the main environmental factors causative of celiac disease (CD) pathogenesis. The incomplete hydrolysis of gliadin by human enzymes during gastrointestinal digestion leads to the generation of peptides able to trigger cytotoxic and inflammatory signals in intestinal epithelial cells of CD patients (1). Clinical manifestations of the disease often include intestinal symptoms and nutrient malabsorption associated with severe mucosal damage (2). Currently, the only available therapy

for CD patients is the adherence to a strict life-long gluten-free diet. However, compliance with this dietary recommendation is complex and other alternative or adjuvant strategies are needed (3).

Alterations in the composition of the gut microbiota of CD patients are characterized by decreased *Bifidobacterium* numbers (4, 5). The association of imbalances in the intestinal microbiota and the positive reported roles that bifidobacteria plays on intestinal health has led to propose the use of probiotics as part of additional and alternative nutritional strategies for improving

the quality of life of CD patients. Certain strains of the *Bifidobacterium* genus effectively reduced *in vitro* the toxicity and inflammatory potential of gliadin-derived peptides (6). The positive effect of bifidobacteria was attributed to their proteolytic capacity on gliadin peptides; however, the effects produced by different bifidobacteria seemed to be strain-dependant demonstrating the most positive effect produced by *B. longum* CECT 7347 strain, which motivated the present study.

Gliadin-derived peptides are thought to stimulate enterocytes (7, 8) and macrophages (9) via specific surface receptors such as the chemokine CXCR3, a 7-transmembrane G-protein, present in the brush border membrane, causing the up-regulation of pro-inflammatory cytokine expression (10, 11). These effects have been linked to the activation of the nuclear transcription factor kappa-B (NF- $\kappa$ B) pathway in the small intestinal mucosa of CD patients (12). After sensing an inflammatory stimulus, the inhibitor I $\kappa$ B is phosphorylated triggering its degradation, thereby allowing the translocation of NF- $\kappa$ B to the nucleus where it induces the expression of a variety of genes involved in inflammation, immune regulation, and cell survival and proliferation (13). Furthermore, alterations in the integrity and function of the intestinal barrier have also been associated to gliadin toxicity (14). Gliadin-derived peptides can

mediate the disorganization of tight junction-related proteins leading to increased intestinal permeability (15), and expression (mRNA) of ionic channels and the chemokine CXCR3 receptor (6, 7). However, there is limited knowledge of the possible role of the interplay between gliadin peptides and intestinal bacteria in the activation of pathogenic or protective responses in the intestine.

The objective of this study was to evaluate the changes in the proteome of Caco-2 cells, widely used as human intestinal model, exposed to gliadin digested *in vitro*, in the presence or absence of *B. longum* CECT 7347, and in response to the bifidobacteria alone. The ultimate goal of the study was to gain a better understanding of early alterations induced by gliadin in enterocytes, and their possible modulation by components of the intestinal microbiota.

## MATERIAL AND METHODS

### Bacterial cultures

*Bifidobacterium longum* CECT 7347 was isolated from faeces of healthy infants as described elsewhere (15). The bacterial cultures were grown in Man-Rogosa-Sharpe broth and agar (Scharlau, Barcelona, Spain) supplemented with 0.05% (w/v) cysteine (MRSC) (Sigma, St. Louis, MO), and incubated at 37°C under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK) for 24 h.

The studies employed bacterial cell suspensions with absorbance of 0.5 ( $\lambda$ , 600 nm) corresponding to  $10^8$  colony forming units (CFU)/mL.

### **Cell culture conditions**

The human colon carcinoma (Caco-2) cell-line was obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17, and used in experiments at passages 33-42. Caco-2 cells were grown in DMEM (AQ-Media, Sigma, USA) containing 4.5 g L<sup>-1</sup> glucose, and supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Biowhittaker, Schiedam, Holland), 10% (v/v) fetal bovine serum (Sigma), 1% (v/v) non-essential amino acids (Biowhittaker), 1% (v/v) L-glutamine (Sigma) and 1% (v/v) antibiotic solution (penicillin, streptomycin and fungizone) (Sigma). Cells were maintained at 37 °C in 5% CO<sub>2</sub>/95% relative humidity and culture medium was changed every 2 days (6).

For experimental studies, Caco-2 cells were seeded at densities of 50,000 cells/cm<sup>2</sup> onto 6-well plates (Costar, Corning, USA). Cell cultures were grown with DMEM, and culture medium was changed every 2 days. Experiments were performed 5 days post seeding. Cell cultures exposed to DMEM were used as controls.

### ***In vitro* digestion of gliadin**

The gastrointestinal digestion process

was simulated as previously described (16), using porcine pepsin (P-7000, Sigma) (800-2500 units/mg protein), pancreatin (P1750, Sigma) (activity 4×USP specifications) and bile (B3883, Sigma). Three mL of a saline solution (140 mM NaCl, 5 mM KCl adjusted to pH 3) was added to aliquots (150 mg) of a commercially available gliadin extract (G3375, Sigma). The mixture was immersed in a water bath (60 °C) for 30 minutes with gentle agitation. Once the samples were at room temperature, gastric and intestinal digestions were conducted on a rocking platform shaker placed in an incubator (37°C/5% CO<sub>2</sub>/95% relative humidity). After the gastric digestion (pepsin in 0.1 M HCl/pH 3/1h), the intestinal digestion (pancreatin-bile in 0.1 M NaHCO<sub>3</sub>/pH 6.8-7.0/2 h) was carried out in the upper chamber of a two-chamber system in 6-well plates formed by fitting the bottom of a Transwell insert ring (Costar) with a 15,000 molecular mass cut-off dialysis membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA, USA). Aliquots (1.5 mL) of the gastro-intestinal digests, inoculated or not with bacterial cell suspensions ( $10^8$  CFU/mL), were loaded into the upper chambers and 1 mL of saline solution (pH 7) was added to the bottom chamber. Afterwards, the inserts were removed and total protein content in the dialysates was quantified using a Lowry method based on a commercial kit (TP0200,

Sigma).

### Toxicity experiments

Caco-2 cell viability in cultures exposed (6 h) to digested gliadin (0.25 mg/mL), inoculated or not with *B. longum* CECT 7347, were determined by the neutral red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) (Sigma) uptake assay (6). The culture medium was removed and kept to determine cytokines as described below. Cells were washed twice with phosphate buffer saline (PBS) at pH 7.2 (Sigma) and NR uptake was measured using a commercial kit (No. 7H092, Sigma) at 540 nm with background subtraction at 690 nm. Control cells exposed to DMEM were analyzed in every assay.

### Analysis of cytokines

Tumor necrosis factor (TNF)- $\alpha$  (eBioscience Inc, San Diego, CA, USA), interleukin (IL)-1 $\beta$  (eBioscience) and IL-10 (eBioscience) were determined in the supernatant from cell cultures by ELISAs according to the manufacturers' instructions. The results of the ELISA assays are expressed as picograms per mL (pg/mL).

### Protein Extraction and 2-DE separation

Caco-2 cells were exposed for 6 hours to digested gliadins (0.25 g/mL), inoculated or not with *B. longum* CECT 7347, or to the bifidobacteria ( $10^8$  CFU/mL). The culture

supernatant was removed and kept to determine cytokines as described below, and cells were washed twice with PBS for proteome analyses (pH 7.2) (Sigma).

Protein extraction was performed as described elsewhere (17) by pipetting with lysis buffer [7 M urea (GE Healthcare, Princeton, USA), 2 M thiourea (GE Healthcare), Nonidet p-40 (USB Corporation, Queveland, USA) (1% v/v), and protease inhibitors (Roche, Complete 04 693 116 001)]. After centrifugation at 4 °C and 11,000 *g* for 10 min, total protein content was determined as previously described. Aliquots (200  $\mu$ g of protein/2  $\mu$ L) were mixed with rehydration solution (340  $\mu$ L) [7 M urea, 2 M thiourea, DTT (Fluka, Arjeplog, Sweden) (0.2%, v/v), Nonidet p-40 (1%, v/v), Pharmalyte 3-10 (GE Healthcare) (1%, v/v) and bromophenol blue] and loaded in the dry polyacrylamide gel strips with an immobilized pH gradient (IPG) (GE Healthcare, Immobiline™ DryStrip pH 3-10 NL, 18 cm). The IPG strips were covered with mineral oil (GE Healthcare) and kept at room temperature overnight. Protein separation according to the isoelectric point (pI) was conducted with an Ettan IPG-Phor II cuvette and the program was visualized through Ettan control software 1.0. Isoelectric focusing (IEF) was carried out under the following conditions: step 1, increase from 0 to 500 V in 5 h; step 2, 500 V for 5 h; step 3, increase from 500 to 3,500 V in

9.5 h; and step 4, 3,500 V for 4 h. Focused IPG strips were equilibrated with equilibration buffer [50 mM Tris-HCl (pH 8.8) (Sigma), SDS (2%, v/v), 6 M urea, glycerol (30%, v/v), dithiothreitol (DTT) (1%, v/v) and iodoacetamide (IAA) (Sigma) (2.5%, w/v)]. The second dimension was run (Ettan Dalt Six cuvette) in pairs side by side. The strips were sealed with an agarose solution 0.5% (w/v) and run overnight at a constant voltage in a polyacrylamide gel (Bio-Rad, California, USA) (acrylamide-bisacrylamide, 12.5%-0.27%, 2.2 C). The analyses were performed in triplicate for each treatment.

### **Tryptic digestion and analysis of protein spots**

Two dimensional (2-D) gels of independent triplicates of each treatment were revealed by silver staining as described elsewhere (18). The stained gel was digitally scanned and spot detection, quantification and analysis were managed using PDQuest software (Bio-Rad). 2-D gel data were normalized by dividing each spot quantity by the total of all of the valid spots in the 2-D gel image to obtain a normalized spot quantity value. For each matched spot, the mean of the values from three 2-D gels was calculated. The differences in protein expression between controls and treated groups were considered statistically significant at  $P < 0.05$  applying the Student's  $t$  test. Gel spots were excised from the gels using biopsy punches. Proteins selected

for analysis were in-gel reduced, alkylated, and digested with trypsin (Promega, Madison, WI). Briefly, spots were washed twice with water, shrunk for 15 min with acetonitrile (ACN) 50% (v/v), and dried in a vacuum centrifuge for 30 min. After reduction with 10 mM DTT in 25 mM  $\text{NH}_4\text{HCO}_3$  for 30 min at 55 °C, the samples were alkylated with 55 mM IAA in 25 mM  $\text{NH}_4\text{HCO}_3$  in ACN 50% (v/v) for 20 min. Then, samples were dehydrated with ACN 100% for 5 min and digested with 12.5 ng/mL sequencing grade trypsin (Promega) in 25 mM  $\text{NH}_4\text{HCO}_3$  (pH 8.5) for at least 16 h at 37 °C.

### **Identification of peptides and database search**

Peptide analyses were performed using an Applied Biosystems 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a 355 nm pulsed nitrogen laser and operated in reflectron positive ion mode as previously reported (19). Briefly, the digested samples were mixed with a 5 mg/mL  $\alpha$ -Cyano-4-hydroxycinnamic acid (CHCA) matrix solution and dissolved in ACN 50% (v/v) with formic acid 0.1% (v/v). The indicated collision cell pressure was increased from  $4.0 \times 10^{-8}$  mbar (no collision gas) to  $6.6 \times 10^{-7}$  mbar. At a resolution above 10,000 in MS mode, the monoisotopic peptide signals could be accurately mass measured (<50 ppm). The mass spectrometry (MS)

and MS/MS spectrum data of tryptic peptides were obtained from MALDI-TOF MS in the reflector mode and used to search for protein candidates in SwissProt and/or National Center for Biotechnology Information (NCBI) databases using MASCOT v 2.3. Only those spots that were found in all the independent replicates were considered for further protein fingerprinting and database interrogation. We determined that a protein was correctly identified if the search results satisfied the following criteria: (i) protein is from the correct species (*Homo sapiens*); (ii) peptide mass tolerance of 50 ppm; (iii) MS/MS ion mass tolerance of 0.25 Da; (iv) allow up to one missed cleavage; (v) variable modifications considered were Cys as *S*-carbamidomethyl derivative and Met in oxidized form.

### Statistical analysis

One-way analysis of variance (ANOVA) and the tukey post test were applied. Statistical significance was established at <0.05 for all comparisons. SPSS v.15 software (SPSS Inc., Chigado, USA) was used for the statistical analysis.

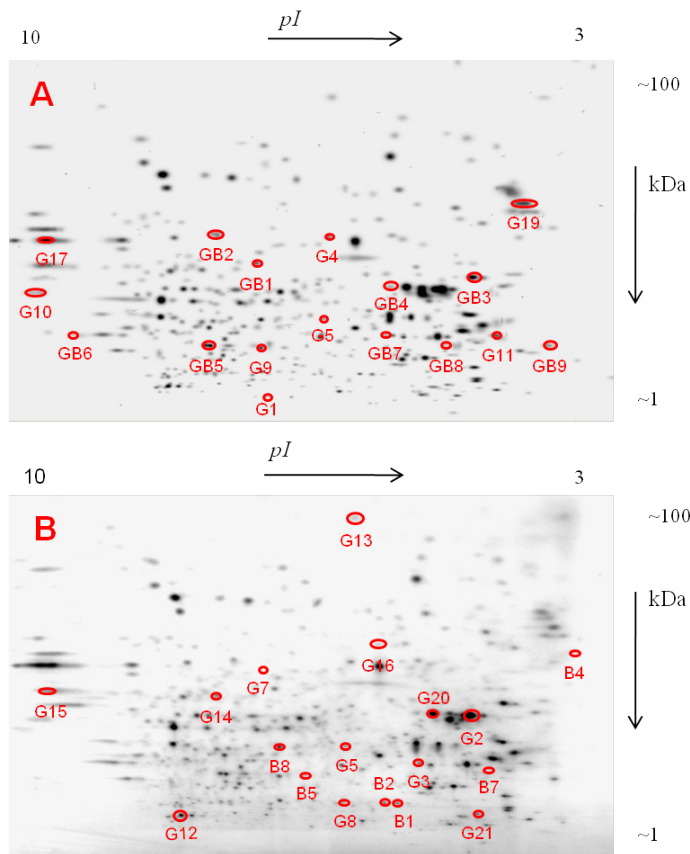
## RESULTS AND DISCUSSION

A recent proteomic approach demonstrated that Caco-2 cells, although they have a tumoral origin, express proteins that are characteristic of human intestinal epithelium at comparable levels to small intestinal

scrapings supporting their use to mimic the small intestine (20). Representative 2-D gels of Caco-2 cell proteomes after the different treatment are shown in **Figure 1**. The proteome of cell cultures exhibited different protein expression patterns (**Table 1**).

In Caco-2 cultures exposed to gliadin digested without *B. longum* CECT 7347 the spots that were up-regulated included proteins that participate in the cellular signalling of gliadin (G12); proteins of the actin filaments and cycle progression (G13), proteins of the outer nuclear membrane that are linked to intermediate filaments (G9) and proteins associated with microtubules (G6 and G5); proteins that regulate apoptosis via apoptotic protease activating factor 1 (APAF-1) and stress-induced apoptosis (G20); and proteins that participate in the cross-talk between intestinal and immune cells (G15 and G17).

The up-regulation detected in regulator G-protein signalling 5 (G12) could reflect the cellular response to counteract the cytotoxic effect of gliadin through their interaction with the CXCR3, a 7-transmembrane G-protein coupled receptor (7). This result is concordant with the significant inhibition of NF- $\kappa$ B activation mediated by both G-protein coupled receptor kinase-2 and -5 reported in other studies (21). It has been also reported that gliadin induced deterioration of actin cytoskeleton and function of intestinal proteins in Caco-2 cells



**Figure 1:** 2-D gel images of the proteins extracted from the Caco-2 cells exposed to gliadin (A) and gliadin inoculated with *B. longum* CECT 7347 (B). The first dimension was run on an immobilized pH gradient (3-10) and the second dimension resolved in a SDS-PAGE 18 cm gel. Proteins were visualized by silver staining. Images show one representative gel of three independent samples. Arrows refer to spots that have been identified by MALDI-TOF MS. The name of the spots corresponds to the nomenclature used in **Table 1**.

(22). The up-regulation of proteins such as Sorting nexin-20 (G15) in response to the gliadin challenge could be related to the activation of interactions between intestinal epithelial cells and immune cells. This protein cycles P-selectin

glycoprotein ligand 1 (PSLG1) into endosomes, via high affinity calcium-dependent interactions with E-, P- and L-selectins (23). Enterocytes, which express human leukocyte antigen (HLA)-DR proteins, are capable of processing transcytosing

**Table 1.** List of identified protein spots of Caco-2 cultures exposed to digested gliadin, inoculated (GB) or not (G) with *B. longum* CECT 7347, and to *B. longum* CECT 7347 ( $10^8$  CFU/mL) alone (B) showing differential production compared to control cultures.

	Protein identified (Mr/pI) <sup>a</sup>	Accession number <sup>b</sup>	Score/peptides matched	(%) Seq Cover	Expression to control <sup>c</sup>
G1	AN1-type zinc finger protein 3 (26023/7.42)	Q9H8U3	28 / 2	14	7.72
G2	Coiled-coil domain-containing protein 29 (32003 /6.41)	Q4UJ79	41 / 3	12	0.01
G3	DNA topoisomerase 3-alpha (114239 /8.69)	Q13472	57 / 9	8	48.69
G4	Keratin, type I cytoskeletal 17 (48361 /4.97)	Q04695	72 / 8	19	0.06
G5	Kinetochore protein Spc25 (26194 /7.71)	Q9HBM1	47 / 4	15	5.73
G6	Microtubule-actin cross-linking factor 1 (623626 /5.27)	Q9UPN3	52 / 8	1	1.31
G7	Myosin-Vc (203978 /7.69)	Q9NQX4	51 / 7	4	19.81
G8	NEDD4-binding protein 3 (60889 /8.33)	O15049	50 / 6	12	1.91
G9	Nesprin-3 (112774 /5.88)	Q6ZMZ3	61 / 9	12	1.25
G10	Probable global transcription activator SNF2L1 (123211 /8.26)	P28370	43 / 7	5	22.40
G11	Protein SSX5 (21647 /9.35)	O60225	37 / 2	14	0.66
G12	Regulator of G-protein signalling 5 (21104 /6.85)	O15539	27 / 3	8	1.50
G13	RhoGEF and PH domain-containing prot 1 (107691 /6.14)	P98174	36 / 3	4	5.80
G14	Secretagoin (32190 /5.25)	O76038	47 / 4	17	1.65
G15	Sorting nexin-20 (36668 /8.94)	Q7Z614	27 / 2	8	3.60
G16	Tetratricopeptide repeat protein 38 (53267 /5.61)	Q5R314	44 / 3	7	2.20
G17	T-cell receptor $\alpha$ chain V region CTL-L17(15545 /8.54)	P04437	41 / 3	14	4.71
G18	Ubiquitin-protein ligase E3A (101593/5.12)	Q05086	42 / 4	5	0.31
G19	UV excision repair protein RAD23 homolog A (39642 /4.56)	P54725	57 / 7	17	0.24
G20	Uveal autoantigen with coiled-coil domains (163545 /6.60)	Q9BZF9	33 / 5	3	3.90
G21	V-set and transmembrane domain-containing prot 1 (26405 /4.98)	Q6UX27	27 / 2	17	0.57
B1	Cell death activator CIDE-A (24899 /9.34)	O60543	41 / 3	21	1.67
B2	Docking protein 6 (38692 / 8.72)	Q6PKX4	31 / 3	10	1.41
B3	Elongation factor 2 (96246 /6.41)	P13639	31 / 3	3	75.70
B4	Leucine-rich repeat-containing prot 45 (76475 /5.95)	Q96CN5	50 / 6	10	0.08
B5	SH3 domain-binding glutamic acid-rich-like protein 2 (12375 /6.29)	Q9UJC5	21 / 1	16	3.26
B6	Spectrin alpha chain (280884 /4.96)	P02549	42 / 5	2	0.09
B7	U6 snRNA-associated Sm-like protein LSm4 (15511/10.02)	Q9Y4Z0	38 / 3	17	2.68
B8	Zinc finger protein 540 (79325/9.58)	Q8NDQ6	54 / 4	5	0.72
GB1	Dynein heavy chain 10, axonemal (517677 /5.64)	Q8IVF4	50 / 10	3	1.38
GB2	EF-hand domain-containing (24092 /8.95)	A8MZ26	44 / 3	14	3.68
GB3	EF-hand domain-containing (67022 /9.51)	Q6NXPO	41 / 4	8	1.68
GB4	Golgin subfamily A member 4 (261892 /5.33)	Q13439	85 / 17	8	0.61
GB5	Myosin-4 (223844 /5.67)	Q9Y623	50 / 8	6	0.33
GB6	RWD domain-containing protein 4A (21238 /5.24)	Q6NW29	36 / 2	14	23.27
GB7	Signal recognition particle 54 kDa protein (55953 /8.87)	P61011	51 / 4	10	229.80
GB8	Trafficking protein particle sub 26 (18199 /8.88)	Q86SZ2	29 / 2	13	4.39
GB9	Zinc finger protein 64 homolog (74565 /8.80)	Q9NTW7	46 / 5	9	472.01

<sup>a</sup>Theoretical molecular mass (Mr, Da) and theoretical *pI* of the corresponding protein based on the SwissProt database. <sup>b</sup>SwissProt accession number. <sup>c</sup>The relative expression to controls was determined by comparing the mean value of each spot to that of the corresponding spots of control cultures and expressed as a ratio. Significant differences in protein expression were calculated from triplicate analyses applying the Student's *t* test ( $p < 0.05$ ).



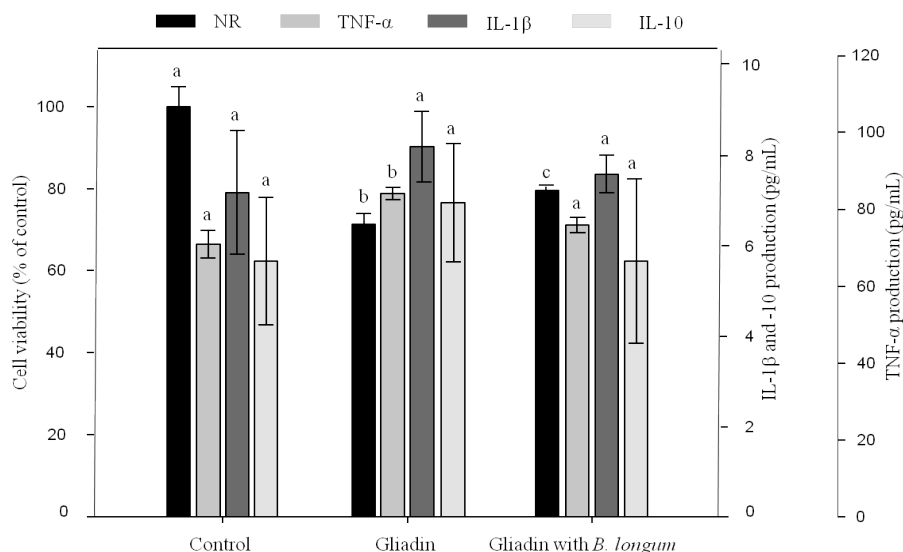
and presenting food antigens to T-lymphocytes of the lamina propria (24). PSGL1 is critical to initial leukocyte capture (25), and could mediate the interactions between enterocytes and T-lymphocytes in response to gliadin. Gliadin exposure also caused up-regulation of the T-cell receptor alpha chain V region CTL-L17 (G17) in Caco-2 cells. Accordingly, the presence of  $\beta/\alpha$  T-cell receptors has been identified in large vacuoles and Golgi complexes of enterocytes from patients with active CD, but not in those from patients on a gluten-free diet (26).

The exposure of Caco-2 cells to digested gliadin also caused the down-regulation of proteins involved in structural functions such as forming stiff bundles of fibres (G2); in DNA-dependent transcription (G11); in post-replication repair functions (G19); in protein synthesis and epithelial cell growth through binding to the adapter protein stratifin and stimulation of the protein kinase B/mammalian target of rapamycin (Akt/mTOR) pathway (G4); and in the cell cycle (G18).

Cytoskeletal (CK) proteins are major structural proteins in eukaryotic cells, which are usually over-expressed in colon carcinoma (27) and gastric cancer cells (28). However, some CK proteins (CK-18 and -19) are down-regulated during apoptosis (29). Apoptosis has been proposed as a major event underlying villous atrophy in CD (30). In the present study, the down-regulation

of gliadin-mediated Keratin type I cytoskeletal 17 (G4) in Caco-2 cells would suggest a pro-apoptotic effect of gliadin. This suggestion is also supported by the up-regulation of Uveal autoantigen with coiled-coil domains (G20), which promotes apoptosis by causing the up-regulation of apoptosome, the down-regulation of lectin galactoside-binding soluble 3 (LGALS3) and the inactivation of NF- $\kappa$ B. However, different *in vitro* studies have reported conflicting results regarding gliadin-induced apoptosis in intestinal cells (6, 31). These studies suggest the likely existence of a threshold for gliadin to cause toxicity. Wheat gliadin (0.5-1.5 mg/mL for 48 h) induced apoptosis of not confluent intestinal cells (Caco-2) by a receptor-mediated (Fas-Fas ligand) pathway (31) similarly to the results reported in the present study. In contrast, in confluent Caco-2 cultures (5 days post-seeding) exposed to digested gliadin (0.25 mg/mL for 15 h), apoptosis was not detected (6). All the aforementioned changes detected in the proteome of Caco-2 cells after gliadin exposure were associated to an inflammatory response, as reflected by increased TNF- $\alpha$  production (**Figure 2**); however, the exposure to digests of gliadin did not cause statistically significant ( $P>0.05$ ) differences in IL-1 $\beta$  and IL-10 production, although the results showed a similar trend.

In cell cultures exposed to digested



**Figure 2.** Effect of digested gliadin, inoculated or not with *B. longum* CECT 7347, on cell viability (Neutral red, NR uptake) and production (pg/mL) of tumor necrosis alpha (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$ , and IL-10. Different superscript letters indicate significant ( $P < 0.05$ ) differences for each treatment.

gliadin inoculated with *B. longum* CECT 7347, changes were detected in nine proteins (**Table 1**). The seven up-regulated proteins might participate in vesicular transport (GB8); in calcium homeostasis (GB2); in DNA binding (GB9); and in ubiquitination in the case of proteins associated with ubiquitin-protein ligase activity (GB6). Ubiquitination is not only important in cellular homeostasis, but also in human disease, ensuring controlled degradation of proteins. Even if the up-regulation of proteins involved in this pathway was able to induce ubiquitination and degradation of the (I $\kappa$ B $\alpha$ ) inhibitor and activate

inflammation via the NF $\kappa$ B pathway, the exposure of Caco-2 cells to gliadin digested with the bifidobacteria was not associated with an inflammatory response (**Figure 2**). Previous data also demonstrate that NF- $\kappa$ B is not activated in intestinal cells exposed to digests of gliadin inoculated with *B. longum* CECT 7347 (6). These results could be explained, at least in part, by the fact that ubiquitin ligases become highly specific by post-translational modification of the substrate proteins, and targets of the ubiquitin ligase also include many other types of proteins, such as cell cycle regulatory proteins and proteins unable to fold properly at

the endoplasmic reticulum. EF-hand domain-containing proteins (GB2), which participate in calcium homeostasis, were also up-regulated under the effects of gliadin digested in the presence of the bifidobacteria. This response could result from the activation of molecular systems controlling cytoplasmic  $\text{Ca}^{+2}$  levels into cells, which could contribute to stabilizing cytoskeletal alterations caused by gliadin in intestinal cells. It has been reported that gliadin-like peptides binding to CXCR3 receptor induced the over-expression of the glutamate receptor (7) coupled to ion channels controlling  $\text{Ca}^{+2}$  influx into cells (32) playing a critical role in the cellular resistance to apoptosis (33). This might suggest that *B. longum* CECT 7347 could reduce gliadin-induced toxicity to intestinal epithelial cells by regulating  $\text{Ca}^{+2}$  homeostasis. This hypothesis could explain the increased viabilities of Caco-2 cells exposed to gliadin digested in the presence of *B. longum* CECT 7347 compared to those not inoculated with the bacterium (**Figure 2**).

It has been suggested that *Bifidobacterium* strains play an important role in health by influencing the host's intestinal barrier functions and the epithelial and immune cell response(s) (15, 34). To test this hypothesis, the influence of *B. longum* CECT 7347 alone on Caco-2 cell proteome was also evaluated. *B. longum* CECT 7347 exposure induced significant changes

in eight proteins, five of which were up-regulated and three were down-regulated (**Table 1**). *B. longum* CECT 7347 induced proteins involved in ribosomal translation (B3 and B7); in cell proliferation (B1); and in bacterial recognition (B4). The up-regulation of proteins involved in ribosomal translation in the presence of *B. longum* CECT 7347 could contribute to the adequate synthesis of proteins in intestinal epithelial cells and thus their rapid turnover. Up-regulation of protein leucine-rich repeat-containing protein 45 (B4) could be involved in the bacterial recognition by Toll-like receptors as part of their leucine-rich domains (35).

The exposure of Caco-2 cells to *B. longum* CECT 7347 also led the down-regulation of the SH3 domain-binding glutamic acid-rich-like protein 2 (B5), zinc finger protein 540 (B8) and spectrin alpha chain (SPTA) (B6). The SPTA is a membrane protein that plays a role in signal transduction, regulating voltage-dependent anion channels (36), which could contribute to gut physiology by modulating electrolyte transport processes through the apical side of enterocytes.

The results from the present study reveal marked changes in the proteome of intestinal epithelial cells in response to their interaction with gliadin-derived peptides. Caco-2 cell cultures exposed to gliadin digested in the absence of *B. longum* CECT 7347 showed

alterations of key proteins involved in CK integrity, cell apoptosis and interactions with immune cells, and significant increases in TNF- $\alpha$  production. In contrast, when gliadin was digested in the presence of *B. longum* CECT 7347 their effect on the proteome was ameliorated, inducing alterations in a lower number of proteins. These proteins were involved in controlling ionic homeostasis, CK integrity and signal transduction. Altogether, the results suggest that *B. longum* CECT 7347 reduces the toxic and inflammatory effects of gliadin-derived peptides, and could contribute to improving the survival and physiological function of intestinal epithelial cells. The significance of these proteomic changes *in vivo* deserves further investigation.

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## Chapter 2

- *Bifidobacterium longum* CECT 7347 modulates immune responses in a gliadin-induced enteropathy animal model





# ***Bifidobacterium longum* CECT 7347 modulates immune responses in a gliadin-induced enteropathy animal model**

Laparra JM, Olivares M, Gallina O, Sanz, Y.

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## ABSTRACT

Coeliac disease (CD) is an autoimmune disorder triggered by gluten proteins (gliadin) that involves innate and adaptive immunity. In this study, we hypothesise that the administration of *Bifidobacterium longum* CECT 7347, previously selected for reducing gliadin immunotoxic effects *in vitro*, could exert protective effects in an animal model of gliadin-induced enteropathy. The effects of this bacterium were evaluated in newborn rats fed gliadin alone or sensitised with interferon (IFN)- $\gamma$  and fed gliadin. Jejunal tissue sections were collected for histological, NF $\kappa$ B mRNA expression and cytokine production analyses. Leukocyte populations and T-cell subsets were analysed in peripheral blood samples. The possible translocation of the bacterium to different organs was determined by plate counting and the composition of the colonic microbiota was quantified by real-time PCR. Feeding gliadin alone reduced enterocyte height and peripheral CD4<sup>+</sup> cells, but increased CD4<sup>+</sup>/Foxp3<sup>+</sup> T and CD8<sup>+</sup> cells, while the simultaneous administration of *B. longum* CECT 7347 exerted opposite effects. Animals sensitised with IFN- $\gamma$  and fed gliadin showed high cellular infiltration, reduced villi width and enterocyte height. Sensitised animals also exhibited increased NF $\kappa$ B mRNA expression and TNF- $\alpha$  production in tissue sections. *B. longum* CECT 7347 administration increased NF $\kappa$ B expression and IL-10, but reduced TNF- $\alpha$ , production in the enteropathy model. In sensitised gliadin-fed animals, CD4<sup>+</sup>, CD4<sup>+</sup>/Foxp3<sup>+</sup> and CD8<sup>+</sup> T cells increased, whereas the administration of *B. longum* CECT 7347 reduced CD4<sup>+</sup> and CD4<sup>+</sup>/Foxp3<sup>+</sup> cell populations and increased CD8<sup>+</sup> T cell populations. The bifidobacterial strain administered represented between 75-95% of the total bifidobacteria isolated from all treated groups, and translocation to organs was not detected. These findings indicate that *B. longum* attenuates the production of inflammatory cytokines and the CD4<sup>+</sup> T-cell mediated immune response in an animal model of gliadin-induced enteropathy.

**Keywords:** Coeliac disease, *Bifidobacterium*, gliadin, weaning rats, enteropathy.

## **INTRODUCTION**

Coeliac disease (CD) is an autoimmune enteropathy triggered by cereal gluten proteins (gliadin) in genetically predisposed individuals (1). In CD patients, peptides resulting from incomplete protein hydrolysis by digestive enzymes cause a deregulated immune response and inflammation. The degree of intestinal inflammation can vary from intraepithelial lymphocytosis to severe infiltration of mononuclear cells in the lamina propria, causing villous atrophy and crypt cell hyperplasia in the small intestine (2). Several attempts have been made to develop animal models that reproduce CD pathogenesis, including the immune response, the mucosal lesions and the symptoms (3-5). The intragastric administration of gliadin to inbred rats after weaning (4) or to immunocompetent mice at 10 days of age failed to reproduce the damage of the intestinal mucosa (6). Human leukocyte antigen (HLA)-DQ8/HCD4 or single HLA-DQ8 transgenic mice sensitised with gluten developed an immune response to gliadin that involved both the adaptive and innate immune system (7, 8) and dependent changes in gut neuromuscular and epithelial secretory function (8), but did not develop a gluten-dependent enteropathy. Nevertheless, repeated oral administration of gliadin to rats, previously sensitised with interferon gamma (IFN- $\gamma$ ) immediately after

birth, caused mucosal lesions characterised by shortening of jejunal villi, crypt hyperplasia, and increased cellular infiltration, including CD8 $\alpha\beta$ <sup>+</sup> and CD4<sup>+</sup> T lymphocytes (9). Activation of CD4<sup>+</sup> T-helper 1 (Th1) cells that produce IFN- $\gamma$  and intraepithelial CD8<sup>+</sup> lymphocytes are responsible for the cytotoxic effects on intestinal epithelium, which in turn could increase passage of gliadin antigens to the lamina propria and further activate the CD4<sup>+</sup> Th1 cell response (10,11). Thus, this model reproduces a CD4<sup>+</sup> T cell mediated enteropathy, defined as hyperplastic-infiltrative (type II), similar to that described in CD patients (9). Undoubtedly, further refinement of the available animal model of CD is desirable, but it is considered appropriate to initially explore pathogenic mechanism and potential pharmaceutical or nutritional interventions (9). The production of T cells with regulatory activity (Tregs) constitutes one of immunosuppressive mechanisms that contribute to intestinal tolerance and prevention of autoimmunity. In particular, natural self-antigen-reactive CD4<sup>+</sup>CD25<sup>+</sup> cells acquired Foxp3 expression, a key marker of the development of regulatory activity, in the thymus and then enter peripheral tissues, where they can suppress the activation of other self-reactive T cells contributing to immune tolerance. These Tregs (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) are particularly increased in the mucosa

and peripheral blood of active CD patients as a consequence of the activation of a regulatory response to counteract the inflammation caused by gluten (12, 13), but their role in animal models of CD has not been studied so far.

In recent years, innate immunity and early interactions of gliadin-derived peptides with intestinal epithelial cells have also been considered critical in the development of the disease. Some gluten peptides can mediate an innate-immune response that involves induction of interleukine (IL)-15 production by epithelial and dendritic cells. IL-15 induces up-regulation of the non-MHC class I receptor NKG2D on intraepithelial lymphocytes (IELs), and its ligand MICA on epithelial cells, that interact and activate cytolytic function on enterocytes (14). The activation of the NF $\kappa$ B pathway in intestinal epithelial cells also mediates the production of other inflammatory cytokines, such as the tumour necrosis factor (TNF)- $\alpha$ , which facilitates the interaction of IELs and intestinal epithelial cells promoting tissue inflammation (15). In germ-free rat pups, colonisation by the whole microbiota has similar effects as administration of gliadin on IEL subpopulations, suggesting that both factors activate common immunological responses that may influence CD development (4). Human studies also report that CD is characterised by imbalances in the composition of the microbiota and,

particularly, reduced numbers of total bifidobacteria and *B. longum* (16). *In vitro* studies have demonstrated that the presence of *B. longum* CECT 7347 during the intestinal digestion of gliadin leads to the generation of different peptide sequences and reduces their toxic and inflammatory effects on intestinal epithelial cells (17). In addition, *B. longum* CECT 7347 has been shown *in vitro* to counteract the inflammatory response induced by the altered faecal microbiota of CD patients in peripheral blood mononuclear cells (18). Yet, the possible *in vivo* effects of this bifidobacterial strain on CD have not been evaluated.

In the light of the evidence available, in this study we hypothesise that the administration of *B. longum* CECT 7347, with immunoregulatory properties and ability to attenuate *in vitro* gliadin toxicity on epithelial cells, could exert protective effects in a model of gliadin-induced enteropathy in weaning rats.

## MATERIAL AND METHODS

### Bacterial strain and culture conditions

*Bifidobacterium longum* CECT 7347 was isolated from faeces of healthy infants as described elsewhere (18). The bacterial cultures were grown in Man-Rogosa-Sharpe agar and broth (Scharlau, Barcelona, Spain) supplemented with 0.05% (w/v) cysteine (MRS-C; Sigma-Aldrich, St.

Louis, USA), and kept at 37 °C in anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK) for 24 h. For animal studies, a pure culture of the strain was grown overnight and used to inoculate fresh MRS-C broth for 22 h. Cells were harvested by centrifugation (6,000 xg for 15 min) at stationary growth phase, washed twice in phosphate buffered saline (PBS, 130 mM sodium chloride and 10 mM sodium phosphate, pH 7.4), and re-suspended in 10% (w/v) hypoallergenic milk-based formula (Nutramigen®, Mead Johnson B.V., Nijmegen, Netherlands). Aliquots of these cell suspensions were frozen in liquid nitrogen and stored at -80 °C until used. The number of live cells after freezing and thawing was determined by plate counting on MRS-C agar after 48 h of incubation, and were expressed as colony-forming units (CFU) per mL. More than 90% of cells were alive upon thawing and no significant differences were found during storage time (4 months). One fresh aliquot was thawed for every new experiment to avoid variability in bacterial cell viability between experiments.

### **Animals and experimental design**

Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of University of Valencia (SCSIE, University of Valencia, Spain) and the protocol was approved by its Ethic Committee.

Experimental animals were female, weaning Wistar rats, provided by the SCSIE. The adult females were date-mated, and fed *ad libitum* with a standard diet (Harlan Bioproducts, Indianapolis, USA). Shortly after spontaneous birth, animals were randomly distributed into seven different groups (n=6 per group): 1) artificially reared (AR) with the hypoallergenic milk-based formula; 2) AR and fed *B. longum* CECT 7347; 3) AR and fed gliadin-derived peptides (GP); 4) AR and fed GP and *B. longum* CECT 7347; 5) AR sensitised with 1,000 U IFN- $\gamma$  administered intraperitoneally immediately after birth; 6) AR sensitised with IFN- $\gamma$  and fed GP; 6) AR sensitised with IFN- $\gamma$  and fed GP and *B. longum* CECT 7347.

Newborn animals were hand-fed (100  $\mu$ L) using a micropipette every 4 hours (4-5 times a day) until the age of 10 days. They were fed with the hypoallergenic milk-based formula for newborns, composed of: 19 g proteins; 34 g fats; 75 g carbohydrates; 680 Kcal; 260 mOsm per L. The bacterium was administered at a concentration of  $6.0 \times 10^7$ - $8.2 \times 10^8$  CFU/day, as determined by plate counting on MRS-C agar, in a single dose during the 10 days. To obtain the GP, a commercially available extract of gliadin (Sigma-Aldrich, St. Louis, USA) was submitted to *in vitro* digestion and then dialysed using a 15 kDa cut-off membrane (17). Weaning rats were fed 50  $\mu$ g gliadin/day in a single dose during

the 10 days and finally they received a provocative dose of gliadin 100  $\mu\text{g}$  ~2 hours before sacrifice.

Changes in body weight were monitored every two days. After treatment, rats were anaesthetised (isofluran) and killed by exsanguination. Whole blood samples were preserved in EDTA-treated tubes to prevent coagulation (at room temperature) for leukocyte analyses. Sections (1 cm) of the proximal jejunum were immersed in RNA *later* buffer (Qiagen, USA) or Krebs's buffer and kept at  $-80\text{ }^{\circ}\text{C}$  for gene expression and cytokine analyses. Liver, spleen and colon content samples were also collected in PBS and immediately used for microbiological analyses by plate culturing.

### **Histologic and morphometric evaluation**

Jejunal tissue sections (1 cm) were fixed in formaldehyde 10% and then sections of 5  $\mu\text{m}$  were stained with haematoxylin-eosin. The samples were analysed with a Nikon Eclipse 90i microscope equipped with a Nikon DS-5Mc digital camera. Photos were analysed with the Nis Elements software (Nikon Instruments Inc., Melville, USA). The parameters analysed included villi width and length and number of infiltrated cells in the lamina propria because their changes characterized the histologic lesions of this enteropathy, and the number and height of enterocytes that provide an

indication of the disorganization of the cellular epithelial layer.

### **Leukocyte counts**

The morphological identification of immune cells was conducted by the May-Grünwald Giemsa's staining procedure. Aliquots (25  $\mu\text{L}$ ) of blood samples were extended on glass slides and were allowed to air-dry. The samples were covered with May-Grünwald's solution (Sigma-Aldrich, St. Louis, USA) for 3 minutes, and afterwards an equal volume of PBS was added and incubated for 3 additional minutes. The preparations were gently rinsed with PBS, and then covered with a dilution (1:20, v/v) of the Giemsa stain-modified solution in PBS for 12 minutes. Finally, the samples were washed, air-dried and analysed using an Olympus BX51 microscope (Madrid, Spain).

### **Lymphocyte phenotyping**

Aliquots (100  $\mu\text{L}$ ) of peripheral blood were mixed with the following fluorochrome-conjugated antibodies: Anti-rat CD45, CD4, CD8 and Foxp3 (eBiosciences, Hattfield, UK). Then samples were prepared for flow cytometry analysis with the Immunoprep kit (Beckman Coulter, USA) according to the manufacturer's instructions, and further analysed on a Modular Flow Cytometer Cell Sorter (MoFlo Sorter, Dakocytomation, USA).

### Real-time reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissue samples with the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was converted to double-stranded cDNA using AMV Reverse Transcriptase (Promega, Madison, USA). PCR was performed with primers designed for the following *Rattus norvegicus* genes: NFκB (forward 5'-CTT CTC GGA GTC CCT CAC TG-3', reverse 5'-CCA ATA GCA GCT GGA AAA GC-3') and β-actin (forward 5'-CTC TTC CAG CCT TCC TTC CT-3'; reverse 5'-TAG AGC CAC CAA TCC ACA CA-3'), the latter used as a housekeeping gene. The PCR mix (20 μL reaction volume) consisted of 7.5 μL SYBR Green I master mix, 1.3 μmol/L primers, and 2.5 μL cDNA. PCR reactions were performed in triplicate in a LightCycler® 480 (Roche) system with the following conditions: 1 cycle at 95 °C for 5 min, 35 cycles at 60 °C for 20 s and 72 °C for 45 s. The relative mRNA expression of the tested gene relative to β-actin expression was calculated using the  $2^{-\Delta\Delta C_p}$  method (19). Samples of each animal tissue were measured in duplicate and gene expression was expressed as fold-change.

### Cytokine protein assay

Jejunal tissue sections (1 cm) were kept in Krebs's buffer (1

ml) supplemented with protease inhibitors (Roche) until analysis. Tumour necrosis factor-α (TNF-α) (Diaclone, Besançon, France), and interleukine (IL)-10 (Abcam, Cambridge, UK) were determined by ELISAs according to the manufacturer's instructions. Prior to cytokine determination, tissue samples were homogenised in cell lysis buffer using a TissueRuptor (Qiagen). Then, samples were centrifuged (1000 xg, 15 min) to get clear supernatants for cytokine determination. The results of the ELISA assay are expressed as picograms per gram of tissue (pg/g).

### Microbiological analyses

Aliquots (50 mg) of different biologic samples (colon content, mesenteric lymph nodes (MLN), spleen and liver) were diluted (1/9) in PBS and decimal dilutions were plated on MRS-C agar supplemented with mupirocin (80 mg/L) (Sigma-Aldrich) and acetic acid (1ml/L) to increase the selectivity of the medium for bifidobacteria. Counts were performed on the highest dilution plates and were expressed as colony forming units (CFU) per gram of faeces. Isolated colonies (8-10) from colon samples were also checked by RAPD PCR analysis to confirm whether the DNA profile of the isolates corresponded with the DNA profile of a pure culture of the administered strain *B. longum* CECT 7347. The random primer 1254 (5'-CCG CAG CCA A-3') was used for



RAPID-PCR analysis as previously described (20). The RAPD-PCR products were visualised on a 1.5% w/v agarose gel after staining with ethidium bromide.

The composition of the microbiota was also analysed by real-time PCR. Samples of colon content were collected, diluted 1/9 in PBS and homogenised thoroughly by agitation in a vortex. Aliquots were used for DNA extraction using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Genus-, group- and species-specific primers were used as described previously to quantify the different bacterial groups of the intestinal microbiota (21, 22). Briefly, PCR reactions were performed in triplicate in a LightCycler<sup>®</sup> 480 (Roche). Each reaction mixture (15 µl) consisted of 7.5 µl of SYBR<sup>®</sup> Green PCR Master Mix (Roche), 3.5 µl of sterile water, 0.75 µl of each of the specific primers at a concentration of 10 µM, and 2.5 µl of template DNA. 16 rRNA gene copy numbers of each bacterial group or species were calculated by comparing the  $C_t$  values obtained with those from a standard curve (23). Standard curves were generated from serial dilutions of a known copy number of the target gene cloned into a plasmid vector. For each reference strain the 16S rRNA gene was cloned into a pGEM-T Easy Vector System (Promega). An *E. coli* strain was transformed with the recombinant plasmids and plasmid

DNA was extracted from *E. coli* by the miniprep method (24). Six non-zero standard concentrations were used to construct the standard curves for each reference strain representing a species or a group and the plasmid DNA concentrations ranged from  $10^4$  to  $10^{10}$  copies of DNA per reaction. Standard curves were constructed by plotting the  $C_t$  values against the logarithm of their initial template copy number. DNA concentration was measured using a NanoDrop<sup>®</sup> and the corresponding copy number was calculated (25). The following reference strains were used as standards: *Bifidobacterium longum* subsp. *longum* CECT 4503, *Bacteroides fragilis* DSMZ 2451; *Clostridium coccooides* DSMZ 933; *C. leptum* DSMZ 935; *Lactobacillus casei* ATCC 393; and *E. coli* CECT 4558.

### Statistical analyses

Statistical analyses were performed using SPSS v.15 software (SPSS Inc., Chicago, IL, USA). For normally distributed data ANOVA and the Student *t* test were applied and, for non-normally distributed data, the Mann-Whitney *U* test was used. Statistical significance was established at  $P < 0.05$  for all comparisons.

## RESULTS

### Body weight and morphometric analyses of jejunal sections

Significant ( $P>0.05$ ) differences in body weight were not detected among the different experimental animal groups (**data not shown**). The animals sensitised with IFN- $\gamma$  and fed gliadin were the only ones that presented signs of diarrhoea. The animal group that was diagnosed with diarrhoea had faecal spots around the anal area and the colon contents recovered after the sacrifice had watery consistency. Morphometric analyses of jejunal tissue sections revealed that animals fed with gliadin alone did not exhibit significant alterations compared to controls, except for decreased ( $P=0.014$ ) enterocyte height (**Table 1**), but this alteration was restored by simultaneous administration of *B. longum* CECT 7347. In animals sensitised with IFN- $\gamma$  and fed gliadin, there was a significant decrease in villi width ( $P=0.048$ ) and enterocyte height ( $P=0.033$ ) and an increase in enterocyte numbers ( $P=0.001$ ) in the apical part of jejunal sections and also higher ( $P=0.001$ ) infiltration of cells in the lamina propria compared to the controls (**Figure 1**). However, these changes were not observed in animals only sensitized with IFN- $\gamma$  but not fed gliadin. *B. longum* CECT 7347 administration increased villi width ( $P=0.004$ ) and enterocyte height ( $P=0.005$ ), partially restoring the alterations detected in animals

sensitised with IFN- $\gamma$  and fed gliadin but did not decrease the cellular infiltration observed in the IFN/gliadin group. Feeding of *B. longum* CECT 7347 alone or together with gliadin increased ( $P=0.016$  and  $P=0.020$ , respectively) villi length when compared to control animals.

### NF $\kappa$ B mRNA expression analysis

Gliadin feeding significantly ( $P=0.013$ ) reduced NF $\kappa$ B mRNA expression, while the simultaneous administration of *B. longum* CECT 7347 restored its levels, reaching similar values as those of controls (**Figure 2**). In animals sensitised with IFN- $\gamma$  and fed gliadin, NF $\kappa$ B expression was markedly increased ( $P<0.001$ ) and the simultaneous administration of *B. longum* CECT 7347 produced even higher NF $\kappa$ B ( $P=0.045$ ) gene expression. Feeding of *B. longum* CECT 7347 alone to weaning animals did not alter the basal expression of this inflammatory marker indicating that the intestinal inflammatory milieu and the simultaneous presence of other stimuli modify the immune effects of this bacterial strain. Sensitisation with IFN- $\gamma$  immediately after birth did not exert a significant effect in comparison with controls.

### Cytokine production

The cytokine concentrations in jejunal tissue sections from different experimental animal groups quantified by ELISA are shown in **Figure 3**. In tissue samples from

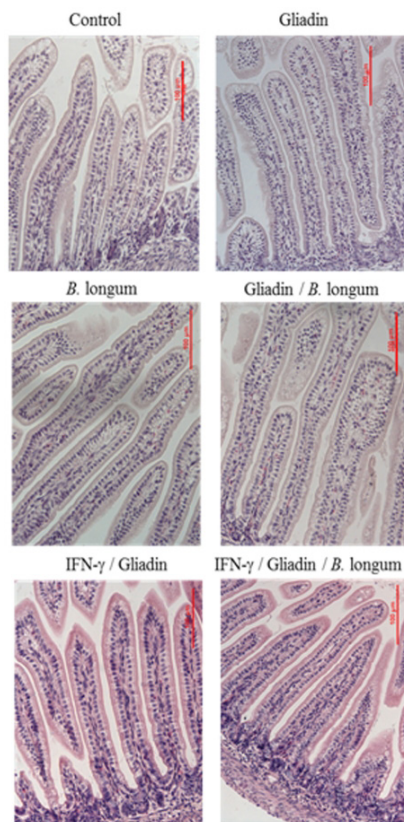
**Table 1.** Morphometric evaluation of jejunal sections.

	Control	Gliadin	<i>B. longum</i>	Gliadin/ <i>B. longum</i>	IFN- $\gamma$	IFN- $\gamma$ /Gliadin	IFN- $\gamma$ /Gliadin/ <i>B. longum</i>
Villi							
width ( $\mu\text{m}$ )	46.15 $\pm$ 7.56 <sup>a</sup>	42.65 $\pm$ 6.39	57.10 $\pm$ 6.23	57.53 $\pm$ 8.36	48.57 $\pm$ 6.35	38.27 $\pm$ 8.46 <sup>a,b</sup>	50.46 $\pm$ 2.61 <sup>a,b</sup>
length ( $\mu\text{m}$ )	193.37 $\pm$ 15.53 <sup>c,d</sup>	156.68 $\pm$ 29.74	255.83 $\pm$ 31.57 <sup>d</sup>	247.44 $\pm$ 51.74 <sup>e</sup>	187.68 $\pm$ 26.10	165.88 $\pm$ 36.62	196.74 $\pm$ 14.00
Infiltrated cells <sup>1</sup>	7.75 $\pm$ 1.25 <sup>e,i,g</sup>	8.80 $\pm$ 1.64	8.12 $\pm$ 0.80	10.24 $\pm$ 1.10 <sup>e</sup>	6.85 $\pm$ 2.12 <sup>h</sup>	11.25 $\pm$ 0.96 <sup>i,h</sup>	10.52 $\pm$ 1.57 <sup>g</sup>
Enterocytes							
height ( $\mu\text{m}$ )	7.04 $\pm$ 1.66 <sup>l,j</sup>	4.29 $\pm$ 1.24 <sup>h,k</sup>	8.32 $\pm$ 1.58	7.71 $\pm$ 1.01 <sup>k</sup>	7.68 $\pm$ 1.92	4.79 $\pm$ 1.03 <sup>l,l</sup>	7.22 $\pm$ 0.96 <sup>l</sup>
counts <sup>2</sup>	4.80 $\pm$ 0.80 <sup>m,n</sup>	6.83 $\pm$ 0.75	5.09 $\pm$ 0.62	6.75 $\pm$ 1.22	5.12 $\pm$ 0.97	8.20 $\pm$ 0.80 <sup>m</sup>	8.01 $\pm$ 0.35 <sup>n</sup>

The results are expressed as mean  $\pm$  standard deviation of 20 independent microscopic fields of each animal (n=6).<sup>a-l</sup> Superscript letters in the same row indicate statistically significant ( $P < 0.05$ ) differences between the pair of samples that has the same letter as determined applying the Student  $t$  test (\*).<sup>1</sup> Number of cells in a 20  $\mu\text{m}^2$  at the lamina propria;

<sup>2</sup> Number of enterocytes along a fixed marker (20  $\mu\text{m}$ ) at the luminal side of the intestinal epithelia.

\*  $P$  values: a, 0.004; b, 0.020; c, 0.016; d, 0.004; e, 0.001; f, 0.007; g, 0.014; h, 0.033; i, 0.005; j, 0.005; k, 0.001; l, 0.001.



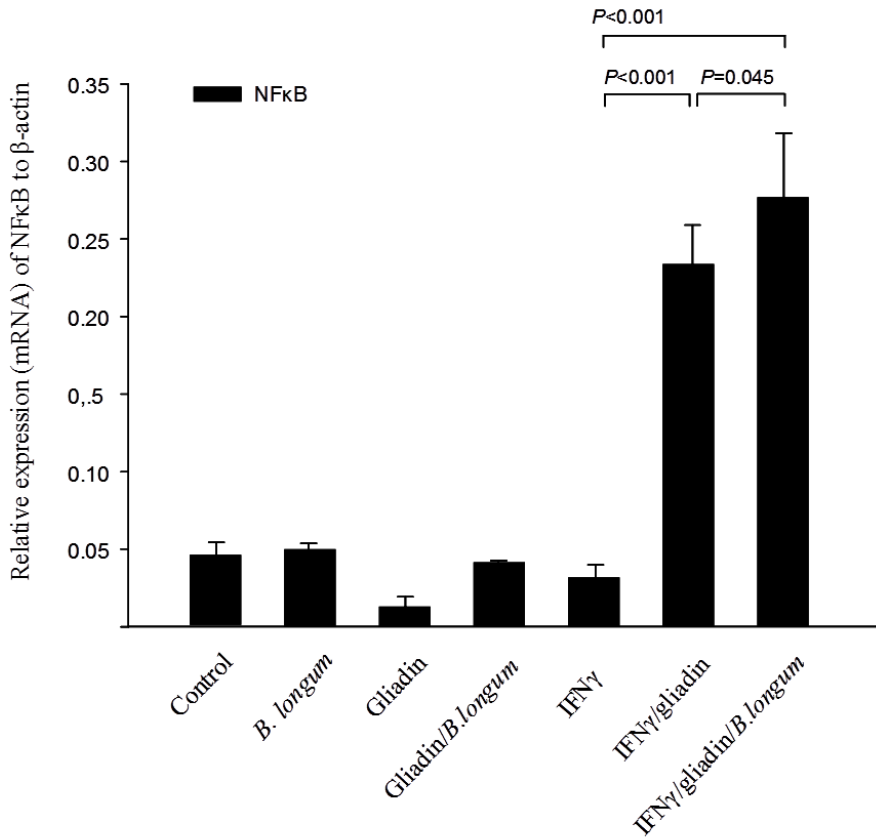
**Figure 1.** Histology of jejunal tissue sections of different experimental rat groups, stained with hematoxylin-eosin showing the different degree of cellular infiltration in the lamina propria of animals sensitized or not with IFN- $\gamma$  and fed gliadins and *B. longum* CECT 7347.

animals fed either gliadin or *B. longum* CECT 7347 the concentration of the inflammatory cytokine TNF- $\alpha$  was not increased, but that of the anti-inflammatory cytokine IL-10 ( $P=0.003$ ,  $P=0.006$ , respectively) did increase in comparison with controls. The simultaneous administration of both gliadin and *B. longum* CECT

7347 caused a significant increase in TNF- $\alpha$  and IL-10 production, in comparison with the control ( $P=0.012$  and  $P=0.028$ , respectively) and with the administration of either gliadin ( $P<0.001$  and  $P=0.017$ , respectively) or *B. longum* CECT 7347 ( $P=0.029$  and  $P=0.001$ , respectively) alone. In animals sensitized with IFN- $\gamma$  and fed gliadin, TNF- $\alpha$  concentrations were significantly ( $P=0.007$ ) increased and, to a lesser extent, also those of IL-10 ( $P=0.008$ ) in comparison with control animals. This effect could be only attributed to the administration of gliadin because animals sensitized with IFN- $\gamma$  but not fed with gliadins did not exhibit significant increases in cytokine production. The administration of *B. longum* CECT 7347 to the enteropathy model significantly reduced TNF- $\alpha$  ( $P=0.003$ ) and increased IL-10 ( $P<0.001$ ) production, indicating its ability to counteract the inflammatory response in the intestinal mucosa.

### Leukocyte count and lymphocyte phenotyping

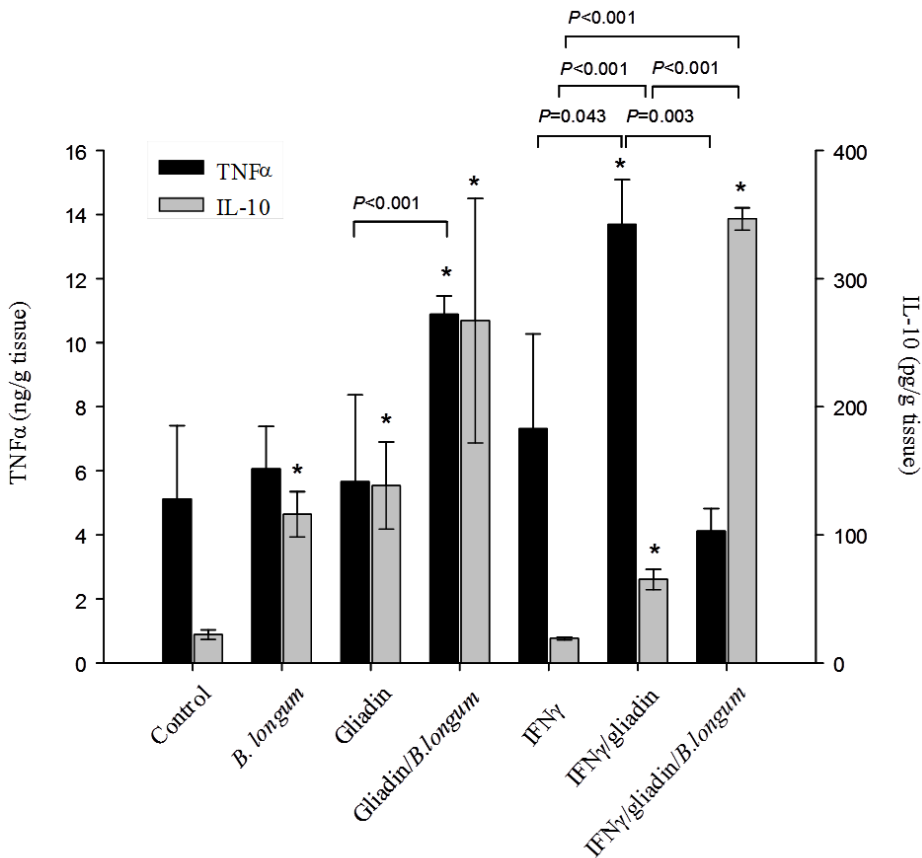
The alterations in peripheral leukocyte populations in different animal groups are shown in **Table 2**. Gliadin administration did not cause significant alterations in the lymphocyte population compared to controls, except for an increase in eosinophils ( $P=0.048$ ). This alteration was restored by simultaneous administration of *B.*



**Figure 2.** Expression (mRNA) of nuclear factor kappa B (NFκB) in jejunal sections of rats after different treatments. The results are expressed as mean  $\pm$  standard deviation (n=6). \*Indicates statistically significant ( $P<0.05$ ) differences relative to the controls; bars indicate significant differences between the specific pair comparisons as determined by applying the Student *t* test.

*longum* CECT 7347 ( $P=0.048$ ). Sensitization of animals with IFN- $\gamma$  did not cause significant changes in leukocyte population, but feeding gliadins to animals sensitised with IFN- $\gamma$  decreased lymphocyte populations significantly ( $P=0.028$ ) in comparison to non-sensitised animals that had been fed gliadin. In contrast, monocyte populations

significantly increased in sensitized and gliadin-fed animals ( $P=0.039$ ) and also in those simultaneously fed *B. longum* CECT 7347 ( $P=0.005$ ) in comparison to controls. The administration of the bifidobacterial strain alone did not significantly modify the leukocyte populations in comparison to controls. In rats, leukocyte counts have been



**Figure 3.** Tumour necrosis factor (TNF)- $\alpha$  and interleukine (IL)-10 production in jejunal tissue sections of rats after different treatments. The results are expressed as mean  $\pm$  standard deviation (n=6). \*Indicates statistically significant ( $P<0.05$ ) differences compared to the controls; bars indicate significant differences between the specific pair comparisons as determined by applying the Student  $t$  test.

reported to be highly variable in the range 6000-18000 leukocytes/ $\mu$ l (26), which is in agreement with the values found in our experimental animal groups. The lymphocyte population was identified with the lymphocyte marker CD45 and further T-cell phenotyping analyses were done within this population (**Figure 4**). Feeding

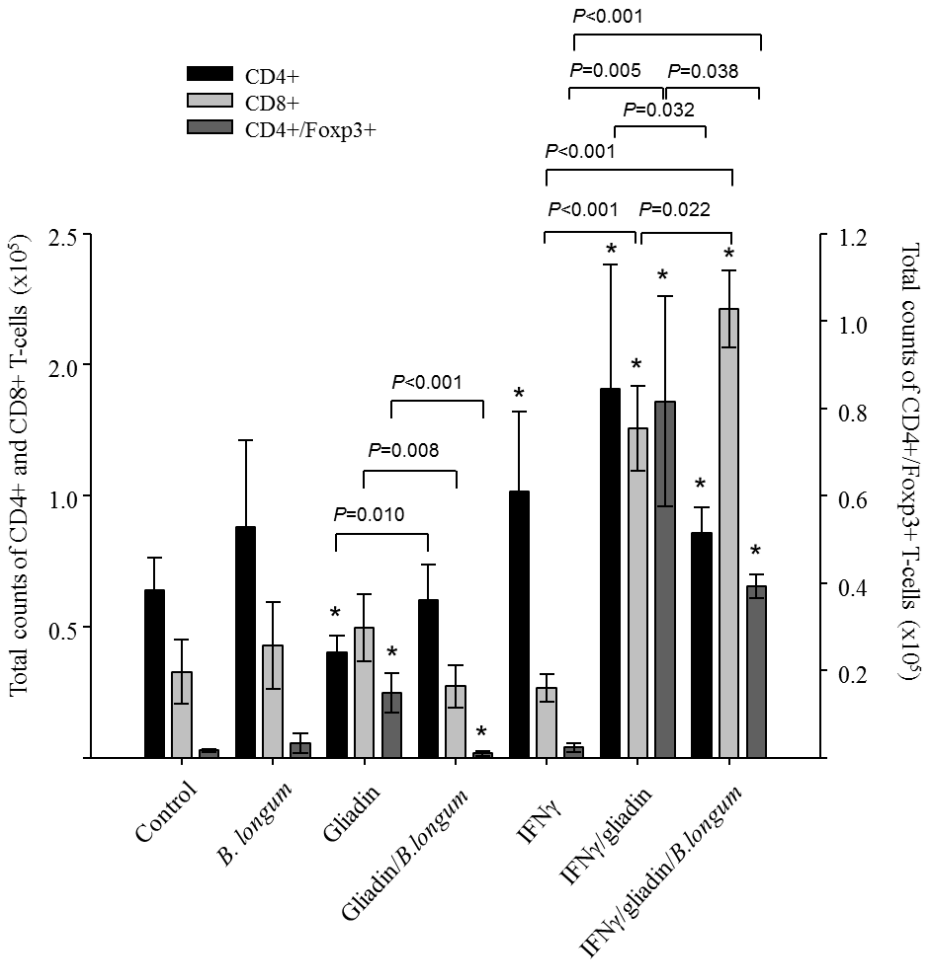
gliadin significantly reduced CD4+ lymphocyte numbers ( $P=0.003$ ) compared to controls, whereas Foxp3+ T cell numbers increased ( $P<0.001$ ). The administration of *B. longum* CECT 7347 together with gliadin exerted the opposite effects in all T-lymphocyte subpopulations, increasing CD4+ cells ( $P=0.010$ ) and reducing CD8+ ( $P=0.008$ ) and

**Table 2.** Leukocyte percentages (%) in peripheral blood of different rat experimental groups.

	<b>Control</b>	<b>Gliadin</b>	<b><i>B. longum</i></b>	<b>Gliadin/<i>B. longum</i></b>	<b>IFN-<math>\gamma</math></b>	<b>IFN-<math>\gamma</math>/ Gliadin</b>	<b>IFN-<math>\gamma</math>/Gliadin /<i>B. longum</i></b>
Neutrophil	17.1 $\pm$ 3.8	18.6 $\pm$ 4.9	20.2 $\pm$ 3.9	19.1 $\pm$ 1.8	17.2 $\pm$ 2.3	22.2 $\pm$ 4.0	18.5 $\pm$ 5.5
Lymphocyte	73.7 $\pm$ 7.1	78.0 $\pm$ 5.6 <sup>a</sup>	72.5 $\pm$ 6.8	73.4 $\pm$ 11.1	73.3 $\pm$ 1.9	66.8 $\pm$ 5.4 <sup>a</sup>	69.2 $\pm$ 4.8
Monocyte	3.3 $\pm$ 0.6 <sup>b,c</sup>	3.6 $\pm$ 0.9	2.9 $\pm$ 1.1	3.4 $\pm$ 1.2	4.1 $\pm$ 1.3	6.0 $\pm$ 1.9 <sup>b</sup>	6.5 $\pm$ 1.5 <sup>c</sup>
Eosinophilo	4.3 $\pm$ 0.7 <sup>d</sup>	5.3 $\pm$ 0.6 <sup>d,e</sup>	5.6 $\pm$ 1.3	2.7 $\pm$ 1.4 <sup>e</sup>	3.3 $\pm$ 1.7	4.2 $\pm$ 1.2	4.9 $\pm$ 1.1
Basophil	1.3 $\pm$ 0.4	1.6 $\pm$ 0.9	1.1 $\pm$ 1.0	1.1 $\pm$ 0.2	2.1 $\pm$ 1.1	0.8 $\pm$ 0.6	0.7 $\pm$ 0.5

The results are expressed as mean  $\pm$  SD (n=6). <sup>a-c</sup> Superscript letters in a same row indicate statistically ( $P < 0.05$ ) significant differences between the pair of samples that has the same letter as determined applying the Student *t* test (\*).

\* *P* values: a, 0.028; b, 0.039; c, 0.005; d, 0.048; e, 0.048



**Figure 4.** Phenotyping of lymphocyte populations in peripheral blood of rats after different treatments. The results are expressed as mean  $\pm$  standard deviation (n=6). \*Indicates statistically significant ( $P < 0.05$ ) differences relative to the controls; bars indicate significant differences between the specific pair comparisons as determined by applying the Student  $t$  test.

Foxp3+ T cells ( $P < 0.001$ ). Animals sensitized with IFN- $\gamma$  exhibited significantly ( $P = 0.041$ ) increased CD4+ cells, but not those of CD8+ and CD4+Foxp3 cells. In addition, in animals sensitised with IFN- $\gamma$  and fed gliadin, there was a much more marked increase in all T-lymphocyte

subpopulations compared to controls. In this model, the administration of *B. longum* CECT 7347 significantly reduced CD4+ ( $P = 0.032$ ) and Foxp3+ T ( $P = 0.038$ ) cell populations, but increased those of CD8+ cells ( $P = 0.022$ ). The administration of *B. longum* CECT



7347 alone did not significantly affect any of the T cell lymphocyte populations analysed.

### **Bacterial translocation and microbiota composition**

Translocation of *Bifidobacterium* spp. to liver, spleen and MLN was not detected by plate counting in any of the treated animal groups. RAPD analyses of colonies isolated from selective media for bifidobacteria present in colon samples indicated that the strain administered represented between 75-95% of the total bifidobacteria. The quantitative analyses of specific bacterial groups by real time-PCR also indicated that the administration of the bifidobacterial strain contributed to an increase in the total gene copies of this bacterial group by at least one logarithmic unit (**Table 3**). Neither feeding gliadin alone nor sensitization with IFN- $\gamma$  alone significantly modified the composition of the microbiota in comparison with controls. In animals sensitised with IFN- $\gamma$  and fed gliadin, significantly higher gene copy numbers of the *Bacteroides fragilis* group were detected in comparison with controls ( $P=0.030$ ) and with rats fed gliadin ( $P=0.020$ ) gliadin plus *B. longum* CECT 7347 ( $P=0.049$ ) and in those sensitized with IFN- $\gamma$  ( $P=0.025$ ). The administration of *B. longum* CECT 7347 did not restore microbiota alterations in the enteropathy model and only contributed to increasing total *Bifidobacterium* gene copy

numbers.

## **DISCUSSION**

This study reports for the first time the effects of feeding a bifidobacterial strain (*B. longum* CECT 7347) at an early postnatal period on the intestinal mucosal architecture and markers of innate and adaptive immunity in an experimental animal model of gliadin-induced enteropathy.

In previous studies, gliadin administration to inbred rats after weaning (4) or adult mice (6) failed to reproduce the enteropathy, probably due to immunocompetence of the experimental animals and lack of gliadin access beyond the epithelial cell layer. Moreover, feeding gliadin even during the early neonatal period was insufficient to cause mucosal damage and significant alterations in epithelium architecture in agreement with our study (4, 27). Sensitisation of animals with IFN- $\gamma$  appeared to be necessary to cause mucosal damage and immunologic changes resembling those observed in human CD (9). IFN- $\gamma$  administration to weaning animals increases macromolecular transport across Peyer's patches (28), and macrophage priming (29) that favours the full establishment of a jejunal mucosal reaction and the instauration of the enteropathy (9). However sensitisation with IFN- $\gamma$  alone did not caused histological alterations in our

**Table 3.** Microbiota composition of colon content determined by real time PCR and expressed as log copy number of 16S rDNA gene per gram of faeces.

Bacterial group	Control	Gliadin	<i>B. longum</i>	Gliadin/ <i>B. longum</i>	IFN- $\gamma$	IFN- $\gamma$ /gliadin	IFN- $\gamma$ /gliadin / <i>B. longum</i>
<i>C. coccoides</i> group	8.99 <sup>a</sup> (5.12 - 9.80)	8.18 <sup>b</sup> (4.30 - 10.38)	8.37 (5.74 - 11.14)	6.75 <sup>c</sup> (4.53 - 9.10)	7.06 <sup>d</sup> (6.94-7.18)	10.74 (4.50 - 11.1)	10.72 <sup>a,b,c,d</sup> (8.90 - 11.09)
<i>C. leptum</i> group	5.34 (5.15 - 6.65)	5.28 (4.04 - 7.57)	5.58 (4.40 - 9.33)	5.33 (4.62 - 5.97)	7.16 (6.07-8.29)	7.84 (5.09 - 9.13)	8.45 (4.53 - 9.12)
<i>Lactobacillus</i> group	9.60 (8.03 - 10.19)	9.76 (9.17 - 9.98)	10.09 (9.69 - 10.39)	10.01 (9.14 - 10.51)	9.24 (8.14-11.10)	9.47 (8.50 - 10.46)	9.65 (8.40 - 10.32)
<i>Enterobacteriaceae</i>	9.04 (8.91 - 9.84)	9.43 (9.12 - 9.77)	9.33 (9.15 - 9.42)	9.24 (8.85 - 9.52)	8.27 (6.58-10.70)	8.89 (7.90 - 9.52)	9.37 (7.45 - 9.91)
<i>Bifidobacterium</i>	6.77 <sup>e,t,g</sup> (3.16 - 8.45)	6.17 <sup>h,i</sup> (3.00 - 9.66)	8.01 <sup>t,k</sup> (5.32 - 10.56)	8.13 <sup>e,j</sup> (7.26 - 8.61)	7.81 <sup>l</sup> (6.97-9.08)	7.00 <sup>h,m</sup> (4.83 - 10.57)	10.18 <sup>e,t,j,k,l,m</sup> (9.29 - 10.59)
<i>Bacteroides fragilis</i> group	7.44 <sup>n</sup> (3.76 - 8.69)	7.13 <sup>o,p</sup> (5.93 - 8.49)	7.49 (3.30 - 11.41)	5.64 <sup>q,r</sup> (2.06 - 8.10)	7.15 <sup>s</sup> (3.60-10.38)	11.07 <sup>h,o,q,s</sup> (9.99 - 11.3)	10.80 <sup>p,t</sup> (7.09 - 11.39)

Data are expressed as median (range of values) (n=6).

<sup>a-n</sup> Superscript letters indicate statistically significant ( $P < 0.05$ ) differences between the pair of samples that has the same letter by applying the Mann-Whitney *U* test.

study, in accordance with previous reports (9). Thus, the animal model used in the present study approaches an intermediate state between proliferative and destructive phases of CD that ultimately lead to complete villous atrophy and disruption of intestinal epithelium integrity, which are major characteristics of fully developed CD (2, 30). *B. longum* CECT 7347 administration to gliadin-fed animals sensitised with IFN- $\gamma$ , partially reduced some of the alterations in jejunal architecture caused by the triggers of the disease, which could theoretically contribute to improving the gut barrier function and preventing gliadin translocation to the submucosa. Similarly, administration of *Lactobacillus casei* ATCC 9595 restored the intestinal damage caused by gliadins in HLA-DQ8 transgenic mice treated with indomethacin (27).

In CD patients, gliadin acts as potent inducer of inflammatory gene expression and cytokine production, including TNF- $\alpha$  and NF $\kappa$ B (31-33). In this study, NF $\kappa$ B expression (mRNA) was increased in animals sensitised with IFN- $\gamma$  and fed gliadin, in agreement with the NF $\kappa$ B activation found in the intestinal mucosa of CD patients (32). NF $\kappa$ B activation has been identified as the mechanism by which gliadin mediates TNF $\alpha$  production in enterocytes (17) and human monocytes (33). Accordingly, TNF- $\alpha$  production was also significantly increased in animals sensitised with IFN- $\gamma$  and fed gliadin.

However, the administration of gliadin alone neither induced NF $\kappa$ B expression nor TNF- $\alpha$  production in comparison with controls. It is possible that gliadin administered alone stimulates a regulatory response reflected in the down-regulation of NF $\kappa$ B mRNA expression and the increased IL-10 production leading to tolerance in these animals, which are not genetically predisposed to the disease. In fact, previous authors also demonstrated that IFN- $\gamma$  administered intraperitoneally was necessary to induce the disease together with oral administration of gliadin (9). In contrast, the simultaneous administration of *B. longum* CECT 7347 and gliadin increased NF $\kappa$ B mRNA expression and cytokine production in comparison with the group only fed gliadin. This could be due to additional interactions of bacterial components with Toll-like receptors (TLRs) that upon ligand binding can also activate the NF $\kappa$ B pathway and cytokine production. Therefore, the results indicate that this bacterial strain caused certain immune activation in the simultaneous presence of gliadin; however, these effects were not significant in comparison with the control group except for cytokine production and were not translated in other pathologic signs. Notably, the administration of *B. longum* CECT 7347 to animals sensitised with IFN- $\gamma$  and fed gliadin reduced TNF- $\alpha$  production and increased IL-

10 production and NF $\kappa$ B expression, triggering an anti-inflammatory and regulatory response. Interestingly, the two treatments IFN- $\gamma$  sensitization plus gliadin feeding and IFN- $\gamma$  sensitization plus gliadin and *B. longum* feeding activated NF $\kappa$ B mRNA expression, but the final effect on cytokine production markedly differed. The effects on these inflammatory markers on our enteropathy model are in agreement with the inflammatory role of gliadins in CD patients and enterocytes previously reported (15, 32). However, an increased in NF $\kappa$ B mRNA expression does not always lead to an inflammatory response because this pathway is regulated at different stages and by diverse mechanisms controlling, for instance, the ubiquitination of the inhibitor I $\kappa$ B, which promotes the translocation of the heterodimer p50/p65 to the nucleus and the final induction of inflammatory cytokines such as TNF- $\alpha$  (34). In this context, it has been reported that some commensal bacterial can induce transient activation or inhibition of the NF $\kappa$ B signalling pathway at different steps that contribute to attenuating and regulating the pro-inflammatory responses. For example, *B. thetaiotaomicron* acts downstream NF $\kappa$ B activation by promoting nuclear export of NF $\kappa$ B subunit relA in complex PPAR- $\gamma$  (35), while other commensal bacterial block NF $\kappa$ B at more proximal steps, inhibiting ubiquitination

and proteolytic inactivation of the endogenous NF $\kappa$ B inhibitor I $\kappa$ B (36). Therefore, it is possible that the *B. longum* strain used in this study only causes a transient activation of NF $\kappa$ B mRNA expression without enhancing the final production of inflammatory mediators such as TNF- $\alpha$  in the enteropathy animal model.

In addition, our results evidence significant differences between the immunomodulatory properties of *B. longum* CECT 7347 and *L. casei* ATCC 9595, since this latter strain was unable to rescue IL-10 production in the enteropathy model of HLA-DQ8 transgenic mice (27). IL-10 production was also stimulated by the administration of *B. longum* CECT 7347 in control animals but not that of TNF- $\alpha$ , which is an additional indication of the anti-inflammatory properties of this strain also in the absence of other stimuli such as gliadin or an inflammatory condition. IL-10 seems to be indispensable for the induction of oral tolerance to dietary antigens, the inhibition of chemokine production and the antigen-presenting capacity of monocytes and macrophages, and induction of the production of soluble antagonists of pro-inflammatory cytokines such as IL-1 and TNF $\alpha$  (37).

Leukocyte counts and phenotyping analyses of T-cell subsets in peripheral blood support that IFN- $\gamma$  sensitisation of weaning animals is effective in stimulating a

T cell-mediated response to orally administered gliadin antigens, partially mimicking the effect in humans. Monocyte numbers were significantly increased in animals sensitised with IFN- $\gamma$  and fed gliadin, which suggests a response to inflammatory signals that could not be significantly reduced by *B. longum* CECT 7347. The data from lymphocyte phenotyping indicated that gliadin alone reduces CD4+ T cells and increases CD4+/Foxp3+ T cells, suggesting a regulatory response in agreement with previous data. Although these changes were reversed by *B. longum* CECT 7347 administration, indicating that the bacterium can induce certain immune activation in an opposite direction, these effects were not significant in comparison with controls. Our study also demonstrates that IFN- $\gamma$  sensitisation, prior to gliadin administration, was necessary to induce an enteropathy mediated by CD4+ T cells, while IFN- $\gamma$  sensitisation alone did not cause significant changes in lymphocyte subpopulations. In the enteropathy model, the changes in CD4+ were also accompanied by an increase in CD4+/Foxp3+ (Tregs) cells, which suggests the development of a counter-regulatory response, as previously reported (7,11). The increased Treg cell numbers is concordant with the increased percentages of circulating regulatory CD4+CD25+Foxp3+ T cells found in untreated, compared to treated (gluten-free diet) CD

patients (12,13). In this study, feeding of *B. longum* CECT 7347 significantly decreased CD4+ and CD4+/Foxp3 Tregs cells in animals sensitised with IFN- $\gamma$ , indicating its ability to counteract the Th1-type inflammatory response triggered by gliadin. However, a recent study has demonstrated that *L. casei* ATCC 9595 administration was unable to significantly modulate CD25+ T cell populations in HLA-DQ8 transgenic mice that had been fed gliadins (27). *B. longum* CECT 7347 also induced CD8+ T cells in this model of enteropathy in agreement with the microbiota-mediated increase in CD8+ lymphocytes previously reported (4); the role of which in this disease remains to be determined.

Microbiota composition of animals sensitised with IFN- $\gamma$  and fed gliadins showed increased gene copy numbers of the *Bacteroides fragilis* group in comparison with control animals; these differences were not associated with gliadin intake and, therefore, could be due to the induced alterations in intestinal epithelium architecture and the underlying inflammation in the enteropathy model (30, 31). This alteration resembles that found in paediatric CD patients that showed increased bacteroides numbers in faeces and duodenal biopsies, but not solely associated with the inflammatory phase of the disease (38, 39). Bifidobacteria and lactobacilli numbers were not related to gliadin intake or the

induced inflammation, in contrast to human data (38, 39). As expected, administration of *B. longum* CECT 7347 led to increased gene copy numbers of total bifidobacteria in the colon, which could be responsible for the biological effects detected on the mucosa and inflammatory markers under gliadin or IFN- $\gamma$  and gliadin administration. Studies *in vitro* and *in situ* have reported positive effects of different *Bifidobacterium* strains in the context of CD, including the reduction of gliadin toxicity (17, 40) and inflammatory potential of gliadin peptides on intestinal cells (17). This study has demonstrated the protective effects of *B. longum* CECT 7347 against the aberrant gliadin response *in vivo*, by reducing inflammatory cytokine production and increasing regulatory cytokine production (IL-10) in the jejunal mucosa, and reduced activation of CD4+ T cells. However, the limitations of the animal model used to fully reproduce the fundamental features of the disease makes necessary to consider the results reposted with caution and, undoubtedly, studies in humans will be necessary to really prove beneficial effects of this bacterium on the disease.

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## Chapter 3

- **Human-milk composition differs in healthy mothers and mothers with celiac disease**
- **The HLA-DQ2 genotype selects for early intestinal microbiota composition in infants at high risk of developing celiac disease**



# Human-milk composition differs in healthy mothers and mothers with celiac diseases

Marra Olivares<sup>1</sup>, Simone Albrecht<sup>2</sup>, Giada De Palma<sup>1</sup>, María Desamparados Ferrer<sup>1</sup>, Gemma Castillejo<sup>3</sup>, Henk A. Schols<sup>2</sup>, Yolanda Sanz<sup>1</sup>

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<sup>1</sup>Microbial Ecology, Nutrition & Health Research Group. Institute of Agrochemistry and Food Technology. National Research Council (IATA-CSIC). Valencia, Spain.<sup>2</sup>Laboratory of Food Chemistry. Wageningen University. Wageningen, The Netherlands.<sup>3</sup>Paediatric Gastroenterology Unit, Hospital Universitari Sant Joan de Reus, Universitat Rovira i Virgili. Tarragona, Spain.



## ABSTRACT

To investigate whether breast-milk composition and microbiota differ in healthy mothers and mothers with celiac disease (CD) to ultimately contribute to identify additional factors determining CD risk. Breast-milk samples from healthy mothers (n=12) and mothers with CD (n=12) were collected. Cytokines and secretory immunoglobulin A (sIgA) were analysed by bead-arrays and flow cytometry and human milk oligosaccharides (HMOs) were assessed by capillary electrophoresis with laser-induced fluorescence (CE-LIF) detection. Breast milk microbiota composition was analysed by conventional and quantitative real-time PCR. Breast-milk from CD mothers showed significantly lower levels of interleukin (IL) 12p70 ( $P < 0.042$ ), transforming growth factor (TGF)- $\beta$ 1 ( $P < 0.018$ ) and sIgA ( $P < 0.003$ ) and almost significantly lower levels of interferon (IFN)- $\gamma$  ( $P < 0.058$ ). Six mothers in each group belonged to the secretor Le(a-b+) type, one to the secretor Le(a-b-) type and five to the non-secretor Le(a+b-) type. CD mothers of non-secretor Le(a+b-) type showed increased Lacto-N-tetraose content ( $P < 0.042$ ) compared with healthy mothers. CD mothers' milk showed reduced gene copy numbers of *Bifidobacterium* spp. ( $P < 0.026$ ) and *B. fragillis* group ( $P < 0.044$ ). CD mothers' breast milk is characterized by a reduced abundance of immunoprotective compounds (TGF- $\beta$ 1 and sIgA) and bifidobacteria. The reduction of these components could theoretically diminish the protective effects of breast-feeding on the child's future risk of developing CD.

**Keywords:** Celiac disease, human-milk, immunity, microbiota.

## INTRODUCTION

Celiac disease (CD) is an autoimmune disorder for which the main genetic determinant (human leukocyte antigen (HLA)-DQ2/8) and environmental trigger (gluten) are known, although they do not fully explain CD onset. Perinatal and early postnatal environmental factors, influencing immune development and gut ecosystem of young children, have also been associated with CD susceptibility (1). Longer breast-feeding and, specially, breast-feeding at the time of gluten introduction seems to reduce the risk of developing CD or at least delay its onset in most case-control observational retrospective studies included in the meta-analysis by Akobeng *et al.* (2). A more recent cross-sectional study performed in two birth cohorts, one born in the so-called “Swedish CD epidemic” (1993) and another born after the epidemic (1997) indicates that reduced CD prevalence (clinically and screening-detected cases) in the second cohort at 12 years of age were related to changes in early feeding practices. These practices included the gradual introduction of gluten-containing foods from 4 months of age simultaneous to breastfeeding (3). However, no protective effect of breast-feeding on CD risk or CD autoimmunity was observed in other prospective studies (4, 5). Duration of breast-feeding could be associated with reduced or delayed gluten

exposure, which could contribute to the protective effect of breast milk against CD development. It is also biologically plausible that breast-milk components (immune mediators, human milk oligosaccharides, etc.) promote oral tolerance to dietary antigens by modulating immune development and function, and infant’s gut microbiota composition (1, 6).

The colonization of the new-born intestine by an adequate consortium of bacteria may contribute to proper development of the host’s immune functions and, conversely, imbalances in microbiota can increase susceptibility to immune-mediated disorders in early and later life. Animal studies have provided direct evidence of the role gut microbial colonization plays in immune developmental processes and the orchestration of appropriate or aberrant immune responses (7). Human observational studies have also reported imbalances in gut microbiota composition that precede the development of specific immune-mediated diseases later in life, thus suggesting causation (8). Although colonization seems to be host-specific, breastfeeding is one of the main environmental factors influencing microbiota composition early in life (9, 10). These effects are thought to be primarily mediated by the presence of oligosaccharides, which are minimally hydrolysed by human enzymes and reach the large intestine, constituting the main substrate for the infants’ gut

microbiota and, particularly, for bifidobacteria (6). In recent years, breast milk has also been considered as a possible source of the bacteria colonizing the infant's gut (10, 11). The demonstration that lactic acid bacteria isolated from breast skin and milk are different, demonstrate their different origin and suggest an endogenous route for breast milk colonization (12). Comparisons between bacterial strains present in mother-infant pair samples also suggest possible bacterial transference from the mother's milk to the infant's gut (10). The mechanism involved in colonization of human milk remains unclear but research reports that dendritic cells are able to capture bacteria from the intestinal lumen via the opening of the tight junctions between epithelial cells, subsequently reaching the mammary gland by the dendritic cells CD18<sup>+</sup> (13).

Human milk is also known to provide many bioactive substances involved not only in immune passive protection, but also in modulation of the neonate immunological development (14). A complex network of chemoattractants and cytokines in human milk are thought to play a role in compensating for the developmental delay of the neonate immune system, and in preventing the development of immune-mediated diseases (15). Human-milk cytokine and secretory immunoglobulin A (sIgA) content have been shown to differ depending on various factors, including the

mother's immune status and dietary content of fatty acids, with potential consequences on infant health (16). The intake of probiotic bacteria has also been related to changes in breast milk composition, including differences in cytokine content (17). Yet the origin of immunoactive components in breast milk and its possible relationship with the breast-milk microbiota composition remain unknown.

The primary objective of the present study was to establish possible relationships between microbiota composition, human milk oligosaccharides (HMO) and immune markers (cytokines and sIgA) in breast milk from healthy mothers and mothers with CD. A wider goal of this research is to gain a greater understanding of how early postnatal environmental factors, and their interaction with host-intrinsic factors, could influence the risk of developing CD.

## MATERIAL AND METHODS

### Collection and processing of samples

Breast-milk samples were collected one month after delivery from 12 healthy mothers and 12 mothers with CD under a gluten-free diet (GFD) for more than 2 years and without disease symptoms. CD was diagnosed according to criteria set by the European Society for Pediatric Gastroenterology, Hepatology

and Nutrition (ESPGHAN) (18). Mothers enrolled in the study were instructed to collect the milk sample in aseptic conditions by manual expression into sterile tubes using sterile gloves at early feed. Samples were immediately stored at -20 °C. Five mL of breast milk were centrifuged (10,000 *xg*, 10 min) and the fatty layer was discarded. We collected the supernatant to determine immune markers and the pellet for bacterial DNA extraction, as described below.

### **Ethical standards**

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Committee on Ethical Practice at the Hospital Universitari Sant Joan (Tarragona, Spain). All the mothers participating in the study gave written informed consent.

### **Cytokine and sIgA quantification**

Supernatant concentration of interleukin (IL)-12p70, IL-10, IL-13, interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$  and transforming growth factor (TGF)- $\beta$ 1 in breast milk were quantified using the Multiple Analyte Detection FlowCytomix<sup>TM</sup> inflammation panel following the manufacturer's instructions (eBioscience, San Diego, USA). Samples and standard curves were analyzed on a FACS-Canto II Deckton Dickinson cytometer (BD

Bioscience). Data were processed using the FlowCytomixPro software (eBioscience). Threshold sensitivities for each cytokine were: 1.5 pg/mL for IL-12p70, 1.6 pg/mL for IFN- $\gamma$ , 1.9 pg/mL for IL-10, 4.5 pg/mL for IL-13, 3.2 pg/mL for TNF- $\alpha$  and 10.0 pg/mL for TGF- $\beta$ 1. The sIgA content was quantified in milk supernatants using a commercial ELISA kit following the manufacturer's instructions (Bethyl, Montgomery, USA).

### **Bacterial DNA isolation and real-time PCR for microbiota analysis**

DNA from breast milk was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Real time PCR analyses were carried out to quantify the content of different bacterial groups using specific primers as previously described (19, 20). PCR amplifications were performed in a LightCycler<sup>®</sup> 480 Real-Time PCR System (Roche, Mannheim, Germany) in a multiwell-plate with 15  $\mu$ L of reaction mixture that consisted of 7.5  $\mu$ L SYBR Green PCR Master Mix (Roche), 3.5  $\mu$ L DNase free water, 0.75  $\mu$ L of each specific primer (10 mM) and 2.5  $\mu$ L of the DNA sample. 16S rRNA gene copy numbers of each bacterial group were calculated by comparing the cycle threshold (Ct) values obtained with those from a standard curve. Standard curves were generated from serial dilutions of a known copy



number of the target gene cloned into a plasmid vector. For each reference strain the 16S rRNA gene was cloned into a pGEM-T Easy Vector System (Promega, Madison, USA). Standard curves were constructed by plotting the Ct values against the logarithm of their initial template copy number using LightCycler® 480 Software, Version 1.5.

To study the prevalence of different *Bifidobacterium* species (*B. bifidum*, *B. breve*, *B. catenolatum*, *B. longum* and *B. infantis*) we used specific primers as described by Matsuki *et al.* (21). PCR products were separated in a 2% (w/v) agarose gel stained with ethidium bromide and we recorded the presence or absence of the amplicon according to molecular mass.

#### **Human milk oligosaccharide extraction from breast-milk samples**

Carbohydrates were extracted from one aliquot (1 mL) of breast milk following Stahl *et al.* (22). The procedure included pasteurization (30 min, 70 °C), defatting at 4 °C and protein precipitation by adding double the amount (v/v) of cold ethanol. The carbohydrates extracted from milk were purified by solid phase extraction on graphitized carbon column cartridges (150 mg bed weight, 4 mL tube size; Alltech, Deerfield, IL) as described previously (23, 24). In short, the cartridges were washed with 80/20 (v/v) acetonitrile (ACN)/water containing 0.1%

(v/v) trifluoroacetic acid (TFA) and later with millipore water. After loading the sample extract onto the cartridge, monomers and lactose were largely removed by elution with an aqueous 5% (v/v) ACN solution. The remaining carbohydrates on the cartridge were eluted with 40/60 (v/v) ACN/water containing 0.05% (v/v) TFA. The solution was dried under a stream of air and the dried sample was then rehydrated with millipore water.

#### **Human milk oligosaccharide determination by capillary electrophoresis-laser-induced fluorescence (CE-LIF)**

For CE-LIF analysis, the carbohydrates present in the breast milk samples were derivatized with the fluorescent 9-aminopyrene-1,4,6-trisulfonate (APTS) overnight at room temperature as reported elsewhere (25). Five nanomole xylose was added as internal standard and mobility marker. CE-LIF was performed on a ProteomeLab PA 800 characterization system (Beckman Coulter, Fullerton, CA), equipped with a laser induced fluorescence detector (LIF) (excitation: 488 nm, emission: 520 nm) (Beckman Coulter) and a polyvinyl alcohol-coated capillary (50 µm x 50.2 cm (Beckman Coulter), detector after 40 cm), kept at 25 °C. Samples were loaded hydrodynamically (4 s at 0.5 psi, representing approx. 14 nL sample solution) into the capillary. Separation was performed

in the reversed polarity mode (30 kV, 20 min) in the 25 mM acetate buffer containing 0.4% polyethylene oxide provided in the ProteomeLab Carbohydrate Labeling and Analysis Kit (Beckman Coulter). Due to the low  $pK_a$  of the sialic acid, residues ( $pK_a$  2.6) the separation buffer was adjusted to pH 2.4 by adding 1.2% (v/v) formic acid. Peaks were integrated manually using Chromeleon software 6.8 (Dionex, Sunnyvale, CA). The HMOs quantified are schematically represented in Table 2.

### Statistical analysis

Data distribution was analysed using Shapiro-Wilk W test (SPSS Statistic 19 v software). Normal distribution was found for cytokines, sIgA and HMO data and the t-test was applied to compare the means of the values of the two groups of mothers. Microbiota data did not show a normal distribution and, therefore, the non-parametric Mann-Whitney U test was applied to compare the medians of the values of the two groups of mothers. Categorical variables (presence or absence) for prevalence of *Bifidobacterium* spp. were analyzed using the Fisher test. Correlations between parameters were determined applying the Pearson coefficient. In all cases, statistically significant differences were established at  $P$  value < 0.05.

## RESULTS

### Demographic and clinical characteristics

The demographic and clinical characteristics of the mothers and their infants are summarized in **Table 1**. No significant differences were detected in maternal age, duration of gestation and mode of delivery. All infants were born at term and their birth weight, length and sex distribution were not significantly different between groups. Therefore, the study groups and their milk samples were comparable.

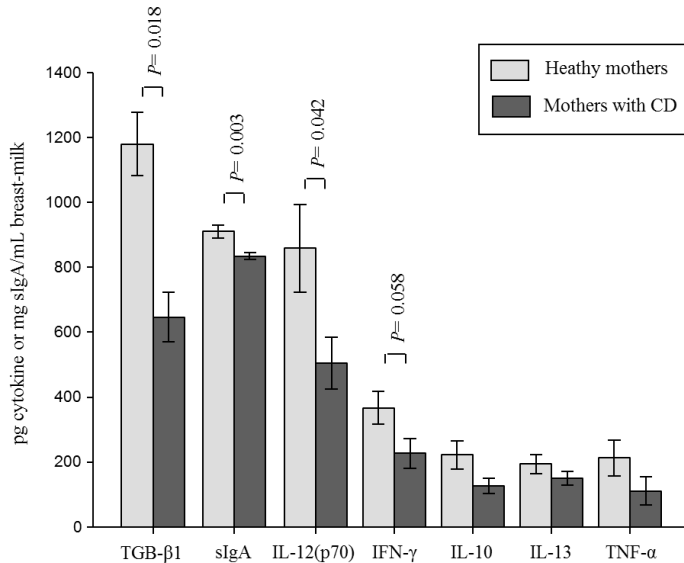
### Immune parameters in breast milk from mothers with CD and healthy mothers

The cytokines and sIgA content in the supernatants of breast milk from 12 healthy mothers and 12 mothers with CD were analyzed (**Figure 1**). The quantifications were positive (above the detection threshold) in 66.7% of cases for IL-13 and TNF- $\alpha$ , and in 37.5% for TGF- $\beta$ 1. The other cytokine quantifications were positive in at least 75% of the samples (data not shown). No differences in cytokine prevalence were observed between groups (data not shown). Overall, mothers with CD had lower cytokine content in breast milk compared with healthy mothers, being statistically significant for IL-12(p70), TGF- $\beta$ 1 and sIgA levels ( $P$ = 0.042, 0.018, 0.003, respectively) and of borderline significance for IFN- $\gamma$  ( $P$ = 0.058).

**Table 1:** Clinical and demographic characteristics of healthy mothers and mothers with celiac disease (CD) and their infants.

Characteristics <sup>a</sup>	Healthy mothers (n=12)		Mothers with CD (n=12)		P-value	
	Mean	SE	Mean	SE		
Mothers	Age	33.2	1.3	34.5	3.6	0.570
	Weeks of gestation	39.1	1.6	38.9	2.4	0.986
	Mode of delivery					
	Vaginal	10 (83.3)		10 (83.3)		0.704
	Caesarean	2 (16.7)		2 (16.7)		
Infants	Birth weight (g)	3650	923	3590	1044	0.704
	Birth length (cm)	48	4	49	4	0.751
	Sex					
	Male	5 (41.7)		4 (33.3)		0.500
Female	7 (58.3)		8 (66.7)			

<sup>a</sup>Data of continuous variables are expressed as mean and standard error (SE) (in brackets) and data of categorical variables are expressed as absolute numbers and percentage (in brackets). Statistically significant differences were established at two-tailed  $P < 0.05$  using the t-test for continuous variables and using the Fisher test for categorical variables.



**Figure 1.** Cytokine and secretory IgA (sIgA) in breast-milk of healthy mothers and mothers with celiac disease (CD). Values are expressed as means and standard error of means. Statistically significant differences were established at two-tailed  $P < 0.050$  using the t-test.

No differences were detected for IL-10 ( $P= 0.094$ ), IL-13 ( $P= 0.228$ ) and TNF- $\alpha$  ( $P= 0.173$ ) between healthy mothers and mothers with CD.

**Oligosaccharide concentrations in breast milk from healthy mothers and mothers with CD**

Healthy mothers and mothers with CD were separated according to their Lewis and secretor profile as:

Le(a-b+)-secretor, Le(a+b-)-non-secretor, Le(a-b)-secretor and Le(a-b)-non-secretor, which depends on fucosyltransferase expression influencing the composition of fucosylated HMOs (26). In both groups, six mothers belonged to the Le(a-b+)-secretor profile, five to the Le(a+b-)-non-secretor, one to Le(a-b)-secretor type and none to the Le(a-b)-non-secretor. **Table 2**

**Table 2.** Human milk oligosaccharides (HMO)<sup>a</sup> analyzed in the present study.

Structure	name	Structure	name
	3'SL		6'SL
	2'FL		3'FL
	DFL		LNT
	LNFP II		LNFP I
	LNFP III		LNDFH I
	F LNHI		F LNHII
	DF LNHI		DF LNHII

<sup>a</sup>Schematic representation of the molecular structure of the human milk oligosaccharides (HMOs) analysed in the present study by capillary electrophoresis-laser-induced fluorescence (CE-FIL). The analyses included two structures of sialyllactoses (SL) 3'SL and 6'SL; two fucosyllactoses (FL) 2'FL and 3'FL; difucosyllactose (DFL); lacto-N-tetraose (LNT); three lacto-N-fucopentaoses (LNFP) LNFP I, LNFP II, LNFP III; one lacto-N-difucosylhexaose (LNDFH) LNDFH I; two fucosyllacto-N-hexaose (F LNHI) F LNHI and F LNHII; and two difucosyllacto-N-hexaose (DF LNHI) DF LNHI and DF LNHII.

summarizes HMO quantification. HMO contents were compared between healthy mothers and mothers with CD of the same secretor status (**Table 3**). In milk of the Le(a+b-)-non-secretor type, higher LNT and LNFPII contents were detected for mothers with CD compared with healthy ones, but only differences in LNT content differed significantly ( $P= 0.042$  and  $0.075$ , respectively). No differences were detected in HMO content between the two groups of mothers with the Le(a-b+)-secretor type.

#### **Breast-milk microbiota**

Breast-milk microbiota composition of healthy mothers and mothers with CD is shown in **Table 4**. Mothers with CD showed a statistically significant reduction in the gene copy numbers of *Bifidobacterium* spp. ( $P= 0.026$ ) and *B. fragillis* group ( $P=0.044$ ) compared with healthy mothers. Of the *Bifidobacterium* species analyzed only *B. bifidum* and *B. breve* were detected but their levels were too low for quantification by real-time PCR. No statistically significant differences were detected in the prevalence of these two species, although, as expected, they tended to be more prevalent in healthy mothers' than in CD mothers' breast milk (**Table 5**).

#### **DISCUSSION**

Here we have demonstrated that breast milk from mothers with CD

and from healthy mothers differs in immune parameters and, albeit to a lower extent, in microbiota and HMO composition. This sheds new light on how breast-feeding may affect the risk of offspring of developing CD, and help explain the controversy across diverse epidemiological studies. Several epidemiological studies have reported that breast feeding provides benefits to the infant's health and may protect against CD, particularly if kept up when gluten is introduced into the diet (2, 3), whereas other studies have not found this association (4, 5). There is also debate as to whether breast-feeding only delays CD onset or actually provides permanent protection, as seems to be the case in the longer study by Ivarsson *et al.* (3). Our case-control study showed TGF- $\beta$ 1 concentration was significantly lower in mothers with CD than in healthy mothers, which may influence the development of the newborn's immune response. TGF- $\beta$  is an important family of growth factors involved in developing oral tolerance and regulating most mechanisms triggered by antigen feeding, as well as in maintaining intestinal immune homeostasis (27). CD patients' pathologic immune response to gluten proteins is characterized by the uncontrolled production of IFN- $\gamma$  by intraepithelial T-cells, which is not sufficiently counteracted by TGF- $\beta$ 1 production, possibly due partly to the inhibitory effect of IL-15 over

**Table 3.** Human milk oligosaccharides (HMOs) content in milk from healthy mothers and mothers with celiac disease (CD).

	HMOs (g/L) <sup>a</sup> in breast-milk											
	Healthy mothers (n=12)						Mothers with CD (n=12)					
	Secretor Le(a-b+)			Non-secretor Le(a+b-)			Secretor Le(a-b+)			Non-secretor Le(a+b-)		
	Mean	SE		Mean	SE		Mean	SE		Mean	SE	
SL	0.38	0.68	0.32	0.81	0.37	0.16	0.49	0.51	0.816	0.103		
FL	2.48	0.66	0.06	0.19	2.54	0.50	0.08	0.22	0.948	0.637		
DFL	0.12	0.35	0.00	0.00	0.17	0.38	0.01	0.01	0.284	-		
LNT	1.23	0.24	1.22	0.29	1.25	0.95	2.00	0.14	0.924	0.042*		
LNFP II	0.27	0.11	0.62	0.16	0.40	0.62	1.13	0.19	0.327	0.075		
LNFP I/III	1.75	0.47	0.38	0.55	2.00	0.38	0.49	0.02	0.690	0.099		
LNDFHI	0.76	0.13	0.00	0.00	1.14	0.20	0.00	0.00	0.153	-		
F LNHI I/I	0.74	0.15	0.68	0.20	0.65	0.68	0.91	0.04	0.596	0.304		
DF LNHI I/II	0.47	0.10	0.40	0.14	0.41	0.33	0.48	0.09	0.570	0.595		

<sup>a</sup>The content (g/L) of different HMO present in breast-milk from healthy mothers and mothers with CD corresponding to the secretor Le(a-b+) or non-secretor Le(a+b-) type was quantified using capillary electrophoresis-laser-induced fluorescence (CE-FIL) and expressed as mean and standard error (SE).

\*Statistically significant differences were established at two-tailed  $P < 0.05$  using the t-test.

**Table 4.** Breast-milk microbiota composition in healthy mothers and mothers with celiac disease (CD).

Bacterial groups	<sup>a</sup> Gene copy numbers/mL of breast-milk		
	Healthy mothers (n=12)	Mothers with CD (n=12)	<i>P</i> -value
<i>Bifidobacterium</i> spp.	2.48 (2.37-2.78)	2.36 (2.29-2.57)	0.026*
<i>B. fragillis</i> group	2.04 (1.89-2.16)	1.96 (1.90-2.03)	0.044*
<i>Enterobacteriaceae</i>	3.25 (3.14-3.38)	3.29 (3.12-3.38)	0.883
<i>Lactobacillus</i> group	3.92 (3.16-4.05)	3.67 (3.29-3.77)	0.140
<i>C. coccoides</i> group	3.05 (2.93-3.43)	3.07 (2.96-3.22)	0.859
<i>Streptococcus</i> group	2.76 (2.70-2.95)	2.99 (2.77-3.04)	0.160
<i>Enterococcus</i> group	2.77 (2.71-2.91)	2.92 (2.71-2.93)	0.093

<sup>a</sup>Data are expressed as median and 25<sup>th</sup> and 75<sup>th</sup> percentiles (in brackets) of log gene copy numbers of each bacterial group. \*Statistically significant differences were established at two-tailed  $P < 0.05$  using the Mann-Whitney *U* test.

**Table 5.** Prevalence of *Bifidobacterium* spp. in breast-milk from healthy mothers and mothers with celiac disease (CD).

	<sup>a</sup> Prevalence of <i>Bifidobacterium</i> spp.		
	Healthy mothers (n=12)	Mothers with CD (n=12)	* <i>P</i> -value
<i>B. bifidum</i>	10/12	7/12	0.185
<i>B. breve</i>	10/12	7/12	0.185
<i>B. catenulatum</i>	0/12	0/12	-
<i>B. longum</i>	0/12	0/12	-
<i>B. infantis</i>	0/12	0/12	-

<sup>a</sup>Prevalence is expressed as positive cases/total cases. Statistically significant differences were established using the Fisher test at two-tailed  $P < 0.050$ .

TGF- $\beta$ 1 signalling (28). Breast milk constitutes an important source of TGF- $\beta$ 1 for the neonate because its expression is absent in the neonatal intestine before day 10, after which it increases progressively (27). The low TGF- $\beta$ 1 levels detected in breast-milk from mother with CD in our study could theoretically limit its protective role in CD prevention. Some authors have described that allergic mothers presented lower levels of TGF- $\beta$ 1 in mature milk (29) or TGF- $\beta$ 2 (17); although inconsistent results have also been reported (30, 31). In the present study, breast-milk of CD mothers was also characterized by a significant decrease in IL-12p70 and important but no significant reductions in IFN- $\gamma$  and IL-10 compared with healthy mothers. IL-12 is one of the main cytokines involved in Th1-type responses, together with IFN- $\gamma$ , which is the main cytokine involved in CD. Therefore, an adverse role of reduced Th1-type pro-inflammatory cytokine concentrations in breast milk of mothers with CD could not be inferred, as anticipated for regulatory cytokine reductions. However, similar changes in cytokine levels in breast milk were associated with increased allergy incidence by Tomicic *et al.* (16). These authors described that reduced concentrations of proinflammatory (IFN- $\gamma$ ) and regulatory (TGF- $\beta$ 2 and IL-10) cytokines and sIgA in breast milk were associated with an increased allergy incidence in the

Swedish population compared to the Estonian population (16). The same trend was reported for allergic mothers' breast milk, where authors interpreted reductions in TGF- $\beta$ 2 and IL-10 as a health risk for the infant (32). The latter is a suppressive cytokine, which plays a key role in intestinal tolerance and immune homeostasis. An animal study reports that transgenic mice with IL-10 gene disruption spontaneously developed enterocolitis at the time of weaning, which was prevented by parenteral administration of IL-10. The study suggests administration of this cytokine with breast milk could play a crucial role in maintaining gut homeostasis (33). However, other authors concluded that IL-10 in breast milk was not required for induction of tolerance and protection from allergic asthma (34). Research has also associated CD with a significant reduction in sIgA levels. This immunoglobulin is produced by breast secretory cells and is able to survive passage through the gastrointestinal tract (35), where it acts as a first-line of defence by interacting with intestinal antigens and microbes. Thus a reduction in sIgA concentrations supplied by breast milk could reduce the infant's mucosal protection, which may precede gluten intolerance. Breast-milk of mothers with CD also showed statistically significant reductions in *Bifidobacterium* spp. and *B. fragillis* gene copy numbers in comparison with healthy mothers'



milk, although differences in *Bifidobacterium* at species level were not detected. Previous studies report that reductions in bifidobacteria content in allergic mothers' breast milk are parallel to reduced infant's faecal bifidobacteria (11).

Probiotic interventions support the hypothesis that gut microbiota plays a role in the immunological properties of breast-milk. For instance, administration of *Lactobacillus rhamnosus* GG was associated with increased TGF- $\beta$ 2 concentration in breast-milk (36) while administration of *L. rhamnosus* GG and *Bifidobacterium lactis* Bb12 together was associated with increased TNF- $\alpha$ , IL-10, IL-4, and IL-2 concentrations (17). However, neither of these studies investigated whether the administered bacteria were detectable in breast-milk samples. The administration of *L. rhamnosus* GG to mothers during pregnancy and breast-feeding was also associated with a reduction in atopic eczema, compared with mothers taking a placebo, but no direct correlation was detected between increased TGF- $\beta$ 2 concentrations and atopic disease or atopy (36). Immunoactive agents present in breast milk can partly come from serum and, therefore, are able to reach the mammary gland via systemic circulation. Probiotics can modify humoral immune mediators via gut stimulation and, therefore, may also influence the composition of immune mediators in breast

milk via a similar mechanism (37). Immune mediators may also be partly derived from the mammary gland epithelial cells (38) and produced locally by breast-milk immune cells (39) where a local stimulatory effect of the breast-milk microbiota on these immune cells is likely. In fact, our study supports this hypothesis, showing parallel reductions in both immune mediators and microbial components in breast-milk of mothers with CD.

The presence and abundance of HMOs in breast milk is genetically determined by glycosyltransferase production in the mammary gland. Most of them are common to all women, with the exception of the fucosyltransferases. The expression of ( $\alpha$ 1,2)-fucosyltransferase (FUT2) and ( $\alpha$ 1,3/4)-fucosyltransferases (FUT3) responsible for the fucosylation of HMOs and others body glycoproteins (e.g. erythrocytes and mucins), depends on the maternal Secretor- (Se) and Lewis (Le) type, respectively (26). Recently, the expression of FUT2, which participates in ABH antigen synthesis in mucus and other secretions, has been associated with bifidobacteria diversity and abundance in the human intestinal tract (40). Likewise, the non-secretor phenotype (characterized by the lack of expression of FUT2 gene) has been associated with susceptibility to CD (41). However, the present study found no differences in the number of cases of the non-secretor

phenotype between healthy and CD mothers, although such differences may not be appreciable due to the limited population size. However, in Le(a+b)-non-secretor mothers, LNT content increased in breast milk of mother with CD compared with healthy mothers' milk. This is the first report of the influence of CD on the presence of HMOs in breast-milk in mothers of a particular secretor status. Mothers secretor status has not been associated with health outcomes in mothers, but with infants' resistance to viral infections (42), which could also constitute risk factors for CD (43).

*Bifidobacterium* spp. can achieve high cell densities using HMOs as sole carbon source, and bifidobacteria are normally abundant in stools from breast-fed infants, therefore HMOs appear to act as a prebiotic substrate, helping to shape the microbiota of the infant's intestinal tract (44). Likewise, HMOs may exert a similar effect on the mammary gland, favouring the presence of bifidobacteria in breast milk. Alternatively, bifidobacteria could be transient bacteria and their levels in the mammary gland may depend on their presence in the maternal intestinal microbiota.

The ability of bifidobacteria to consume HMOs is species-dependent. For example, research reports *B. longum* subsp. *longum* DJO10A and *B. breve* ATCC15700 consumed only a portion of a single, nonfucosylated/nonsialylated HMO species (LNnT), while *B.*

*longum* subsp. *infantis* ATCC15697 consumed oligosaccharides with a low degree of polymerization ( $\leq 7$ ) (44). Accordingly, differences in HMO composition would result in differences in *Bifidobacterium* species composition in human milk but we did not find such differences. Our finding is supported by a previous study that reported differences in infants' microbiota only for *B. adolescentis* and *B. catenulatum* abundances, analysed by denaturing gradient gel electrophoresis (DGGE) in infants fed with milk group 4 (Le(a-b)-non-secretor) according to classification by Thurl *et al.* (45), which only represent 1% of general population (46). This could be explained by the fact that different bacterial species develop different strategies to use HMOs and by the existence of cross-feeding mechanisms among them which overall enable their growth.

The study reported herein has some limitations partly related to its exploratory nature, including a small population size, lack of connexion with clinical outcomes in the offspring and lack of control of mother anthropometric measures and diet, which might also influence the breast milk composition. In particular, it has been recently described that women on a GFD reduce the dietary protein and fibre intakes and increase the fat intake (50), which could also have an impact on breast milk composition. Breastfeeding during the first months

of life has been associated with a protective effect against asthma (47, 48), atopic dermatitis (49) and CD (2) although not all study results are consistent. Compositional differences between breast milk of mothers with allergic disease and controls have also been reported although their relationships with health outcomes are controversial yet. The results presented here show differences between breast milk composition from healthy mothers and mothers with CD in terms of immune mediators, microbiota and HMOs. It can be speculated that such differences could influence the protective effects of breastfeeding on infant health, and partly explain controversy across studies. Nevertheless, further prospective studies are needed to reveal whether differences in breast-milk composition ultimately influence the risk of offspring of developing CD.

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# The HLA-DQ2 genotype selects for early intestinal microbiota composition in infants at high risk of developing celiac disease

Marta Olivares<sup>1</sup>, Alexander Neef<sup>1</sup>, Gemma Castillejo<sup>2</sup>, Giada De Palma<sup>1</sup>, Vicente Varea<sup>3</sup>, Amalia Capilla<sup>4</sup>, Francesc Palau<sup>4</sup>, Esther Nova<sup>5</sup>, Ascensión Marcos<sup>5</sup>, Isabel Polanco<sup>6</sup>, Carmen Ribes-Koninckx<sup>7</sup>, Luis Ortigosa<sup>8</sup>, Luis Izquierdo<sup>1</sup>, Yolanda Sanz<sup>1</sup>

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<sup>1</sup>Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas (IATA-CSIC), Valencia, Spain. <sup>2</sup>Hospital Universitario Sant Joan de Reus, Tarragona, Spain. <sup>3</sup>Gastroenterología, Nutrición y Hepatología Pediátrica. Hospital Universitario Sant Joan de Deu and Unidad de Gastroenterología Pediátrica del Institut Dexeus, Barcelona, Spain. <sup>4</sup> Centro de Investigación Príncipe Felipe (CIPF) and IBV-CSIC Associated Unit, CIBER de Enfermedades Raras (CIBERER), Valencia, Spain. <sup>5</sup>Dept. Metabolismo y Nutrición. ICTAN-CSIC, Madrid, Spain. <sup>6</sup>Servicio de Gastroenterología y Nutrición Pediátrica, Hospital Universitario La Paz, Madrid, Spain. <sup>7</sup>Unidad de Gastroenterología Pediátrica, Hospital Universitario La Fe, Valencia, Spain. <sup>8</sup>Unidad de Gastroenterología, Hepatología y Nutrición Pediátrica, Hospital Universitario Nuestra Señora de Candelaria, Santa Cruz de Tenerife, Canarias, Spain.





## ABSTRACT

Intestinal dysbiosis has been associated with celiac disease (CD), but whether the alterations are cause or consequence of the disease is unknown. This study investigated whether the HLA-DQ2 genotype is an independent factor influencing the early gut microbiota composition of healthy infants at family risk of CD. As part of a larger prospective study, a subset (n=22) of exclusively breast-fed and vaginally delivered infants with either high genetic risk (HLA-DQ2 carriers) or low genetic risk (non-HLA-DQ2/8 carriers) of developing CD were selected from a cohort of healthy infants with at least one first-degree relative with CD. Infant fecal microbiota was analyzed by 16S rRNA gene pyrosequencing and real time quantitative PCR. Infants with a high genetic risk had significantly higher proportions of Firmicutes and Proteobacteria and lower proportions of Actinobacteria compared to low risk infants. At genus level, high risk infants had a significant less *Bifidobacterium* and unclassified Bifidobacteriaceae proportions and more *Corynebacterium*, *Gemella*, *Clostridium* sensu stricto, unclassified Clostridiaceae, unclassified Enterobacteriaceae and *Raoultella* proportions. Quantitative real time PCR also revealed lower numbers of *Bifidobacterium* spp. in infants with high genetic risk than in those of low genetic risk. In high risk infants negative correlations were identified between *Bifidobacterium* spp. and several genera of Proteobacteria (*Escherichia/Shigella*) and Firmicutes (*Clostridium*). The genotype of infants at family risk of developing CD, carrying the HLA-DQ2 haplotypes, influences the early gut microbiota composition. This finding suggests that a specific disease-biased host genotype may also select for the first gut colonizers and could contribute to determining disease risk.

**Keywords:** celiac disease, HLA-DQ genotype, microbiota, pyrosequencing, real-time PCR

## INTRODUCTION

Celiac disease (CD) is a chronic intestinal inflammatory disorder caused by a deregulated immune response to cereal gluten proteins of wheat, barley and rye in genetically predisposed individuals. The expression of the Human Leukocyte Antigen (HLA) Class II molecules DQ2 and DQ8, coded by the DQA1 and DQB1 genes, is strongly associated with susceptibility to CD. The HLA-DQA1\*05:01 and DQB1\*02:01 alleles forming the particular DQ2.5 haplotype confer high susceptibility to CD (1). Susceptibility to CD is increased in those homozygous subjects with this haplotype in *cis*, or those carrying the DQ2.2 haplotype (HLA-DQA1\*02:01 and DQB1\*02:02) in *trans* with the DQ7.5 haplotype (HLA-DQA1\*05:05 and DQB1\*03:01). (2). In CD patients, gluten peptides are deaminated by tissue transglutaminase in the lamina propria and recognized by dendritic cells expressing HLA-DQ2/DQ8 molecules that mediate the typical Th1 response of the disease, producing mainly interferon (IFN)- $\gamma$  (3). Most patients are carriers of the HLA-DQ2/DQ8 genes but these genes are also present in about 40% of the general population, and only a small percentage (2-5%) develops CD (4, 5). This indicates that the HLA-DQ genotype is necessary but not solely responsible for the development of the disease. Gluten

is the main environmental trigger of CD but its intake does not fully explain the onset and clinical expression of the disease. In recent years, other environmental factors influencing the early gut microbiota composition such as type of delivery and milk-feeding, intestinal infections and antibiotic intake, have also been associated with the risk of developing CD (6-11).

Colonization of the newborn intestine is thought to contribute to proper development of the host's immune function and to determine susceptibility to immune-mediated disorders in early and later life (12, 13). Most studies in CD patients report imbalances in the intestinal microbiota (14) with a few exceptions (15, 16). In our own studies, the microbiota of CD patients was characterized by decreased numbers of *Bifidobacterium* spp. and increased numbers of *Bacteroides* spp. in feces and intestinal biopsies (17, 18). Intestinal dysbiosis was not completely restored after adherence to a gluten-free diet suggesting that some changes in microbiota are not secondary to the inflammatory milieu of the active phase of the disease, but could play a primary role in predisposition to CD development. To date, we have a limited understanding of the host genotype's influence on the intestinal microbiota composition, but a few studies have been done in relation to chronic inflammatory bowel disorders (19-21). In the case of CD,

there is only one study in a cohort of infants at family risk of developing the disease that has reported reliable associations between the HLA-DQ genotype and the composition of the intestinal microbiota, assessed by fluorescent *in situ* hybridization (FISH) and real-time polymerase chain reaction (PCR) (22-23). Nevertheless, these preliminary associations could be confounded by diverse environmental variables (e.g. type of milk-feeding, type of delivery, etc). Furthermore, the abovementioned studies were limited by their use of molecular techniques, which can only detect a small number of intestinal bacterial groups for which primers and probes are available. However, this problem can be now overcome by analyzing 16S rRNA gene fragments from the whole fecal DNA (metagenome) by next generation sequencing (NGS) techniques, which help to study the intestinal ecosystem in greater depth (24).

The objective of this study was to characterize the microbiota of exclusively breast-fed and vaginally delivered infants at family risk of developing CD to reveal possible associations between the HLA-DQ2 genotype, the early microbiota composition and the ecological interactions between relevant taxa. The broader aim is to gain a greater understanding of the genetic and environmental factors influencing the early colonization process of the newborn intestine, and their impact

on the risk of developing CD.

## MATERIALS AND METHODS

### Subjects and sampling

A subset (n=22) of one-month-old, exclusively breast-fed and vaginally delivered infants with either high genetic risk (HLA-DQ2 genotype, including homozygous HLA-DQ2.5 or heterozygous DQ2.5/DQ2.2 and DQ2.2/DQ7.5 carriers) or low genetic risk (non-HLA-DQ2/8 genotype) of developing CD were selected from a cohort of healthy infants with at least one first-degree relative with CD participating in a larger prospective study (23). The risk of developing CD was determined by PCR-SSP DQB1 and DQA1 typing as previously described (23). The group of 11 infants classified as high risk (HR) represented the highest probability (>20%) of developing CD and included those carrying the DQ2.5 haplotype (DQA1\*05:01-DQB1\*02:01) in homozygosis and the DQ2.5/DQ2.2 or the DQ2.2/DQ7.5 haplotypes in heterozygosis (**Supplementary table 1**). The low risk (LR) group included those individuals with other common genotypes unassociated with CD, thus having the lowest probability (<1%) of developing CD. None of the infants included in the study received antibiotics during the sampling period.

The study was approved by the local Ethic Committees and written

informed consent was obtained from the parents of infants included in the study.

### **DNA extraction**

For DNA extraction, 0.2 g of fecal sample was homogenized in 15 mL of phosphate buffer saline (PBS) (130 mM sodium chloride, 10 mM sodium phosphate buffered saline, pH 7.4). Afterwards, the suspension was filtered through a 100 µm nylon filter, washed twice with PBS and re-suspended in tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.0 [HCl]). One mL of the suspension was added to 2 mL of lysozyme (2.5 mg/mL) and incubated at 37 °C for 1 hour. After adding 0.67 mg of proteinase K, the mix was incubated at 55 °C for 10 min. A volume of 400 µL 10% (w/v) sodium dodecyl sulfate (SDS) was added to the mix and samples were incubated at 55 °C for 1 hour under gentle shaking. One aliquot of the mix was used for DNA extraction following the DNeasy Blood and Tissue kit protocol (Qiagen, Hilden, Germany)

### **Sequencing the 16S rDNA amplicons**

The extracted metagenomic DNAs were used to amplify the V5 and V6 hypervariable regions of the 16S rRNA using the primers 784F 5'-AGGATTAGATACCCTGGTA-3' and 1061R 5'-CRRCACGAGCTGACGAC-3' by PCR, as previously described (25). The forward primer contained the sequence of the

Titanium A adaptor 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3' and the barcode sequence. For each sample, a PCR mix of 100 µL was prepared containing 1× PCR buffer, 2U of KAPA HiFi Hotstart polymerase blend and deoxynucleotide triphosphates (dNTPs) (Kapa biosystems, Wilmington, USA), 300 nM primers (Eurogentec, Liege, Belgium), and 60 ng gDNA. Thermal cycling consisted of initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 98 °C for 20 s, annealing at 56 °C for 40 s, and extension at 72 °C for 20 s, with a final extension of 5 min at 72 °C. Then 3 µl of PCR product were added to a new PCR mix (identical to the first round of PCR) for the nested PCR of 15 cycles. Amplicons were visualized on 1% agarose gels using GelGreen Nucleic Acid gel stain in 1x tris-acetate-EDTA (TAE) buffer (Biotium, Hayward, USA) and were cleaned using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, USA) according to the manufacturer's instructions. Amplicon DNA concentrations were determined using the Quant-iT PicoGreen dsDNA reagent and kit (Life Tech, Carlsbad, USA) following the manufacturer's instructions. Assays were carried out using 2 µL cleaned PCR product in a total reaction volume of 200 µL in black, 96-well microtiter plates. Following quantitation, cleaned amplicons were combined in equimolar ratios

into a single tube. The final pool of DNA was eluted in a volume of 100  $\mu$ L nuclease-free water, purified using Agencourt Ampure XP Purification systems (Agencourt Biosciences Corporation-Beckman Coulter, Beverly, USA) and then resuspended in 100  $\mu$ L of TE 1x. The concentration of the purified pooled DNA was determined using the Quant-iT PicoGreen dsDNA reagent and kit (Life Tech, Carlsbad, USA) following the manufacturer's instructions. Pyrosequencing was carried out using primer A on a 454 Life Sciences Genome Sequencer FLX instrument (Roche, Basel, Switzerland) with titanium chemistry. 16S rDNA amplicons were sequenced by DNA Vision Agrifood S.A. (Liège, Belgium).

### Sequence and clustering analysis

Original reads were filtered by length ( $> 240$  bp) and quality (average Phred value  $> 25$ ) and then for chimeras using UCHIME (26) resulting in  $12,188 \pm 3,191$  sequence reads per sample on average. Reads were identified at phylum, family and genus level at an 80% confidence level using the Ribosomal Database Project (RDP) multiclassifier tool. Rarefaction curves and cluster analysis were calculated with the mothur package using 1000 randomizations (27). For analysis of Clostridia sequences the Living Tree Project dataset available from [www.arb-silva.de](http://www.arb-silva.de) was used and the respective reads were subjected to

a BlastN search against a database of microbial 16S rRNA gene sequences (SILVA 111, [www.arb-silva.de](http://www.arb-silva.de)).

Similarity of the subject's microbiota was evaluated by cluster analysis of Bray-Curtis distances between individuals, considering the relative abundances of different genera. Decimal logarithms of raw abundance data were classified according to an arithmetic progression in a scale from 0 to 7 (28). Logarithms were obtained adding 1 to each raw value, 0 corresponded to absence of the genera and 7 was fixed as the maximum raw datum considering all genera and individuals. Bray-Curtis distances between subjects were computed and agglomerative nesting cluster (AgNes function of the *cluster* package of R) analysis was applied to obtain the distances (28). Weighted UniFrac analysis was performed with a set of 258 dereplicated sequences with abundances of at least 100 reads (26) using QIIME and the FastUniFrac tool at the URL <http://unifrac.colorado.edu>.

### Richness and diversity index

Microbial biodiversity and richness were analyzed for each sample. Both parameters are based on operational taxonomic units (OTUs), which are clusters of reads defined by their inter-distance using the Chao index. Richness is directly related to the number of observed OTUs whereas the Shannon diversity index depends on the distribution of sequence abundances in the observed OTUs.

The Simpson index is a measure of the probability of randomly resampling an OTU. Microbial richness, Shannon, and Simpson indices were calculated with the *mothur* package (27).

### Correlation analyses

To study possible ecological interactions between different bacterial taxa, we established correlations within the bacterial groups identified in the low and high genetic risk groups of infants. We considered only those genera present in at least nine of the 11 infants in each group. The weighted number of reads for each infant was expressed as a logarithm and cases with double zero (absence of both bacteria compared) were dismissed. The significance ( $P$  value) of the correlation coefficients between abundance of any two genera was estimated by a permutation test, performing 1,000 simulations for each coefficient (29). Only positive correlations with  $|1-P| < 0.100$  and negative correlations with  $P < 0.100$  were considered. Additionally, we considered possible correlations among the genera *Bifidobacterium*, *Corynebacterium*, *Gemella*, *Clostridium* sensu stricto and *Raoultella* because they enabled us to discriminate between both groups of infants in previous analyses.

### Real time quantitative PCR (qPCR)

DNA was amplified using group-

and genus-specific primers as described previously (30, 31) to quantify different bacterial groups in the intestinal microbiota. Each reaction mixture consisted of 7.5  $\mu\text{L}$  of SYBR Green PCR Master Mix (Roche), 3.5  $\mu\text{L}$  of DNase RNase free water, 0.75  $\mu\text{L}$  of each of the specific primers (10  $\mu\text{M}$ ) (Isogen, Barcelona, Spain), and 2.5  $\mu\text{L}$  of template DNA. PCR amplification and detection were performed using a Light Cycler LC480 (Roche). Gene copy numbers of each bacterial group were calculated by comparing the cycle threshold (Ct) values obtained with those from a standard curve. Standard curves were generated from serial dilutions of a known copy number of the 16S rRNA gene cloned into a pGEM-T Easy Vector System (Promega). *E. coli* DH5 $\alpha$  was transformed with the recombinant plasmids and plasmid DNA was extracted by the miniprep method (32).

### Data processing and statistical analyses

Of the demographic data, the categorical variables (number of first-degree relatives with CD) were analysed using the Chi-Square test and the continuous variables (size, weight and weeks of gestation at birth) using a t-test since data distribution was normal as assessed with Shapiro-Wilk W test (SPSS software V19). Data of microbiota composition was not normally distributed as assessed by the same test. Comparisons of data

from qPCR were done by applying the non-parametric Mann-Whitney *U* test (SPSS software V19). Read numbers for each taxon obtained for each infant by pyrosequencing were weighted using the mean values of the total reads for all 22 individuals. The recalculated numbers of reads assigned to each taxon for the high ( $n=11$ ) and low genetic risk ( $n=11$ ) groups of infants were compared using permutation analyses (DAAG package of R software). To compare diversity indices we implemented the R-software Wilcoxon test. Correlations between the data obtained by pyrosequencing and qPCR were analyzed using Pearson correlation coefficient (SPSS software V19). In all cases, statistically significant differences were established at  $P < 0.050$ .

## RESULTS

### Characteristics of the infants included in the study

The demographic characteristics and the HLA genotype of the study infants are included in **Table 1**. No statistical differences ( $P > 0.050$ ) were observed in size, weight and weeks of gestation at birth or in the number of first-degree relatives with CD (mother, father, brother or/and sister) between the two infant groups.

### Microbiota composition by 16S rRNA gene sequencing

The pyrosequencing analysis

detected sequences belonging to four phyla in all samples: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (**Figure 1a**). The intestinal microbiota of infants with low genetic risk of developing CD was characterized by a very high proportion of Actinobacteria (mean [SD] 79.6 [19.1]%), a moderate proportion of Firmicutes (14.9 [16.6]%) and a low proportion of Proteobacteria and Bacteroidetes (3.3 [7.3] and 2.2 [6.2]%, respectively). Infants at high genetic risk of developing CD showed a more heterogeneous and evenly distributed microbiota among the phyla Actinobacteria, Firmicutes and Proteobacteria (36.1 [39.5], 38.2 [28.2] and 22.6 [28.8]%, respectively), with a small proportion of Bacteroidetes (3.0 [9.9]%). High SD values revealed notable inter-individual variability of the microbiota composition (**Figure 1b**). Infants with a high risk of developing CD showed significantly higher proportions of Firmicutes ( $P=0.026$ ) and Proteobacteria ( $P=0.039$ ) and a lower proportion of Actinobacteria ( $P=0.005$ ) than those with low genetic risk. The high risk group of infants also presented higher proportions of Bacteroidetes than the low risk group (3.0 [9.9] versus 2.2 [6.2]%) but the differences were not statistically significant ( $P=0.954$ ).

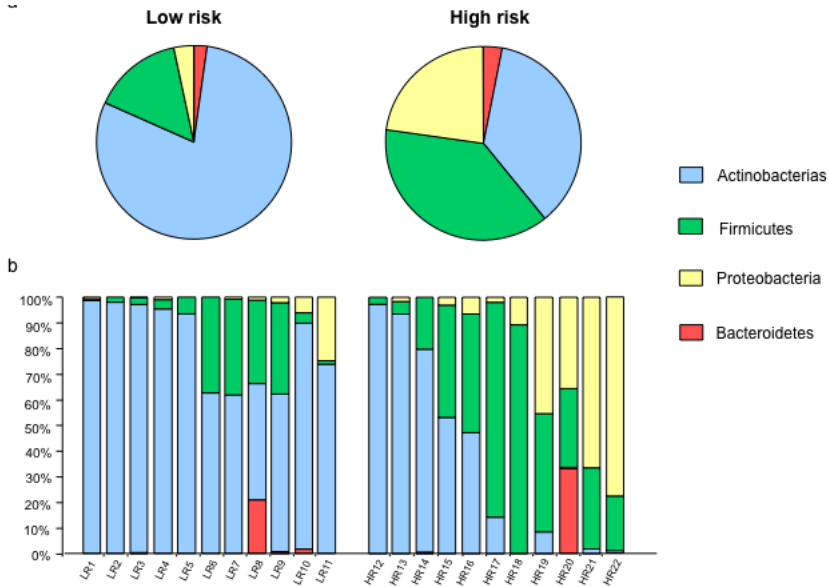
In **Figure 2** is shown a schematic representation of the principal families characterizing the microbiota in the low and high genetic risk

**Table 1:** Demographic characteristics and genotype of the infants included in the study.

ID	First-degree relatives with CD	Genotype *	Size (cm)	Weight (g)	Gestation (weeks)	** Hospital
LR1	brother	DQ7.5/DQ5.1	47.0	3060	40	1
LR2	brother	DQ5.1/(DQ8)	52.1	3850	40	2
LR3	sister	(DQ8)/(DQ5)	54.0	4720	39	3
LR4	sister	DQ5/DQ7.5	51.3	4120	40	1
LR5	sister	DQ5.1/DQ7.5	52.0	3980	40	3
LR6	sister	DQ7/(DQ5)	49.0	4350	41	1
LR7	brother	DQ5/DQ5.3	50.4	4255	35	2
LR8	brother	DQ6.1/DQ4.2	53.1	3750	38	1
LR9	mother	DQ5.1/DQ5.1	53.5	4600	40	2
LR10	sister	DQ6/DQ8	52.0	4290	38	4
LR11	mother	DQ2/DQ4	51.0	4130	39	4
Mean (SE)			51.4 (0.6)	4100.5 (136.2)	39.1 (0.5)	
HR12	sister	DQ2.5/DQ2.5	51.7	3800	41	2
HR13	sister	DQ2.5/DQ2.5	50.0	4210	40	1
HR14	sister	DQ2.5/DQ2.5	50.0	4260	40	2
HR15	father	DQ2.5/DQ2.5	51.5	3890	39	2
HR16	brother	DQ2.5/DQ2.5	53.7	4050	40	2
HR17	sister	DQ2.5/DQ2.5	54.0	3680	39	1
HR18	mother + sister	DQ2.5/DQ2.2	53.5	4860	40	2
HR19	mother	DQ2.2/DQ7.5	48.8	3800	38	2
HR20	sister	DQ2.5/DQ2.2	49.0	3680	40	3
HR21	mother	DQ2.5/DQ2.2	52.6	3900	39	4
HR22	brother	DQ2.5/DQ2.2	54.1	4730	37	4
Mean (SE)			51.7 (0.6)	4078.2 (121.6)	39.4 (0.3)	
<b>P value #</b>	0.590		0.717	0.904	0.654	

\* Displayed in parentheses are genotypes inferred from the DQB1 allele. \*\* 1-Hospital La Paz (Madrid); 2-Sant Joan de Reus (Tarragona); 3- Hospital Niño Jesús (Madrid); 4-Nuestra Señora de la Candelaria (Canarias) # Differences for the categorical variables between the low (LR) and high risk (HR) infant groups were analyzed using the Chi-Square test and for the continuous variables with the t-test. In all cases differences were established at a  $P$  value < 0.050.

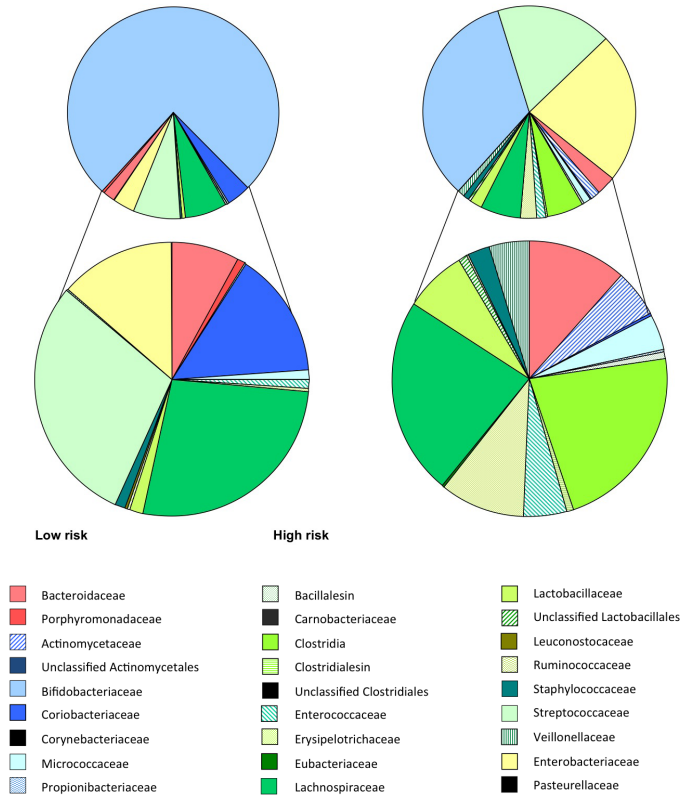




**Figure 1:** Microbiota analysis by pyrosequencing the V5 and V6 hypervariable regions of the 16S rRNA. Mean distribution of the four phyla Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes detected in the high genetic risk (HR) and low genetic risk (LR) groups of infants (a) and their corresponding individual percentages in each infant (b).

groups of infants. In infants with low genetic risk of developing CD the majority (96.4% of sequence reads) of the total bacterial population belonged to five families: Bifidobacteriaceae, Streptococcaceae, Lachnospiraceae, Coriobacteriaceae and Enterobacteriaceae. In infants with high genetic risk of developing CD, the majority (95.9% of sequence reads) of the total bacterial population belonged to ten families comprising: Bifidobacteriaceae, Enterobacteriaceae, Streptococcaceae, Lachnospiraceae, Clostridiaceae 1, Bacteroidaceae, Erysipelotrichaceae, Lactobacillaceae, Actinomycetaceae and Enterococcaceae. In infants

with low genetic risk of developing CD, sequences belonging to the phyla Actinobacteria and Firmicutes were mainly represented by the families Bifidobacteriaceae, and Lachnospiraceae plus Streptococcaceae, respectively. However, in infants with high genetic risk of developing CD, sequences belonging to the phyla Actinobacteria, Proteobacteria and Firmicutes were mainly represented by the families Bifidobacteriaceae, Enterobacteriaceae and Lachnospiraceae, Streptococcaceae plus Clostridiaceae, respectively. Infants with a high risk of developing CD had significantly higher abundance of Clostridiaceae 1 ( $P <$



**Figure 2:** Percentages of the average values of the different families detected in the high and low genetic risk groups of infants. The families are represented with different shades and patterns indicating that they belong to the phyla Actinobacteria (blue), Firmicutes (green), Proteobacteria (yellow) and Bacteroidetes (red). Families shaded black in the figure had abundances below 0.1% and thus their shares are not visible.

0.001), Bacillales Incertae Sedis XI ( $P= 0.009$ ), Enterobacteriaceae ( $P= 0.039$ ), Corynebacteriaceae ( $P= 0.050$ ) and unclassified Clostridiaceae ( $P= 0.012$ ); and lower abundance of Bifidobacteriaceae ( $P= 0.010$ ) than those with low genetic risk.

At genus level, high risk infants had significantly increased proportions of *Corynebacterium* ( $P= 0.050$ ), *Gemella*

( $P= 0.009$ ), *Clostridium* sensu stricto ( $P< 0.001$ ), *Raoultella* ( $P= 0.035$ ), unclassified Clostridiaceae ( $P= 0.013$ ) and unclassified Enterobacteriaceae ( $P= 0.009$ ) and decreased proportions of *Bifidobacterium* ( $P= 0.010$ ) and unclassified Bifidobacteriaceae ( $P= 0.011$ ) compared to low risk infants (**Table 2**).

AgNes cluster dendrogram based on Bray-Curtis distances showed that at

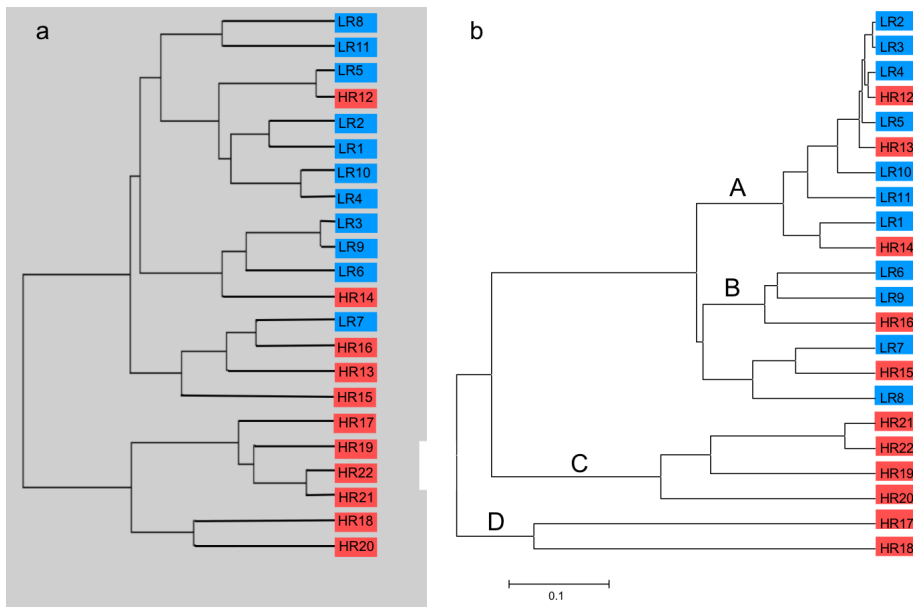
**Table 2:** Mean, standard deviation, median, Q25, Q75 values and ranges of the number of reads in each infants weighted using the total number of reads of all the infants

GENUS level	LOW RISK						HIGH RISK						P
	Mean	SD	Median	Q25	Q75	Range	Mean	SD	Median	Q25	Q75	Range	
<i>Bacteroides</i>	226.61	674.77	0	0	0	0-2253	351.49	1163.33	0	0	0	0-3859	0.945
<i>Parabacteroides</i>	25.39	59.98	0	0	0	0-192	0.11	0.36	0	0	0	0-1	0.214
<i>Bifidobacterium</i>	8811.79	2834.90	10287	6853	10287	3399-11479	3903.72	4777.42	19	6	19	1-11395	<b>0.010</b>
<i>Parascardovia</i>	5.57	18.48	0	0	0	0-61	0.07	0.24	0	0	0	0-1	1.000
Unclassified Bifidobacteriaceae	81.71	45.10	89	46	90	0-157	27.26	43.46	0	0	0	0-143	<b>0.011</b>
<i>Protonibacterium</i>	2.82	8.97	0	0	0	0-30	4.64	14.62	0	0	0	0-49	0.738
<i>Roibia</i>	27.43	45.06	1	0	1	0-131	126.92	276.16	35	2	35	0-942	0.225
<i>Corynebacterium</i>	0.44	0.62	0	0	0	0-1	3.23	6.13	1	0	1	0-21	<b>0.050</b>
<i>Actinobaculum</i>	0.11	0.37	0	0	0	0-1	19.39	64.32	0.	0	0	0-213	1.000
<i>Actinomyces</i>	9.56	27.79	0	0	0	0-93	146.83	438.71	4	0	4	1468-0	0.351
<i>Eggerthella</i>	3.44	6.08	0	0	0	0-18	3.17	10.52	0	0	0	0-35	0.935
<i>Atopobium</i>	2.62	7.37	0	0	0	0-25	1.29	2.13	0	0	0	0-6	0.913
<i>Collinsella</i>	412.68	1130.39	0	0	0	0-3789	0.65	1.05	0	0	0	0-3	0.214
<i>Streptococcus</i>	833.16	1520.25	174	82	173	4-4029	2024.71	3446.25	371	80	371	29-10243	0.351
Unclassified Streptococcaceae	7.72	15.94	0	0	0	0-41	8.19	13.67	3	0	3	0-43	0.939
<i>Lactococcus</i>	2.04	5.15	0	0	0	0-17	5.80	16.83	1	0	1	0-56	0.704
<i>Lactobacillus</i>	41.65	80.71	11	0	11	0-276	212.42	463.85	1	0	1	0-1531	0.279
Unclassified Lactobacillaceae	0.32	1.07	0	0	0	0-4	2.01	4.20	0	0	0	0-14	0.270
<i>Enterococcus</i>	30.18	49.47	2	0	2	0-119	139.15	274.49	16	7	16	0-931	0.175
Unclassified Enterococcaceae	2.06	3.55	0	0	0	0-9	6.37	12.51	1	0	1	0-42	0.358
<i>Leuconostoc</i>	3.25	10.36	0	0	0	0-34	0.00	0.00	0	0	0	0-0	0.476
<i>Fructobacillus</i>	1.88	6.24	0	0	0	0-21	0.00	0.00	0	0	0	0-0	1.000
<i>Granulicatella</i>	0.52	1.73	0	0	0	0-6	3.59	11.90	0	0	0	0-39	1.000
<i>Staphylococcus</i>	32.05	64.21	5	0	5	0-205	74.62	82.23	41	5	41	0-194	0.194
<i>Genella</i>	0.32	0.76	0	0	0	0-2	23.85	54.89	8	0	8	0-186	<b>0.009</b>
<i>Anaerococcus</i>	0.00	0.00	0	0	0	0-0	26.55	87.77	0	0	0	0-291	0.476
<i>Lachnospiraceae incertae sedis</i>	34.92	86.34	0	0	0	0-278	114.64	364.60	0	0	0	0-1213	0.807
<i>Blautia</i>	47.11	155.88	0	0	0	0-517	0.00	0.00	0	0	0	0-0	0.476
Unclassified Lachnospiraceae	561.95	1274.64	1	0	1	0-3714	591.07	1266.05	1	0	1	0-4239	0.995
<i>Clostridium XIVb</i>	0.27	0.91	0	0	0	0-3	1.18	3.62	0	0	0	0-12	0.738
<i>Dorea</i>	121.16	401.85	0	0	0	0-1333	0.21	0.71	0	0	0	0-2	1.000
<i>Clostridium sensu stricto</i>	0.00	0.00	0	0	0	0-0	642.65	1079.04	103	27	103	0-3308	<b>&lt;0.001</b>
<i>Clostridium IV</i>	0.00	0.00	0	0	0	0-0	12.48	41.38	0	0	0	0-137	1.000
<i>Clostridium XVIII</i>	6.85	15.01	0	0	0	0-38	306.12	1009.16	0	0	0	0-3349	0.843
Unclassified Clostridiaceae	0.00	0.00	0	0	0	0-0	18.64	38.45	1	0	1	0-110	<b>0.013</b>
Unclassified Eubacteriaceae	0.00	0.00	0	0	0	0-0	10.28	34.11	0	0	0	0-113	1.000
<i>Veillonella</i>	2.23	6.79	0	0	0	0-23	137.45	439.45	1	0	1	0-1462	0.225
<i>Escherichia/Shigella</i>	345.32	828.59	46	8	46	0-2790	1950.50	3355.77	79	2	79	0-8941	0.151
<i>Klebsiella</i>	2.55	7.18	0	0	0	0-24	426.16	1395.66	0	0	0	0-4634	0.269
<i>Serratia</i>	0.82	2.73	0	0	0	0-9	5.72	18.15	0	0	0	0-60	0.738
<i>Raoultella</i>	0.00	0.00	0	0	0	0-0	20.52	42.06	0	0	0	0-134	<b>0.035</b>
Unclassified Enterobacteriaceae	40.73	55.09	3	1	3	0-135	247.94	261.58	179	59	179	1-657	<b>0.009</b>

Statistical differences between the two risk groups were established at  $P < 0.050$  using permutation analyses.

genus level the microbiota of infants can be partially differentiated based on their HLA-DQ genotype and cluster together with a few exceptions (**Figure 3**). A weighted UniFrac analysis clustered the microbiota according to predominant bacterial genera in each case. Consistent with the AgNes clustering the basic differentiation was between samples

containing or not bifidobacteria. Six high risk infants dominated either by more than 40% Enterobacteriaceae reads (*Escherichia* or *Klebsiella*, subcluster C) or by more than 50% *Streptococcus* (subcluster D) and both not containing bifidobacteria ( $\leq 0.2\%$ ) grouped with each other. A large group of samples (subcluster A) comprising seven from low risk



**Figure 3:** (a) AgNes dendrogram shows the clustering of the infants based on Bray-Curtis distances calculated on the basis of the abundance data (number of 16S rRNA gene sequence reads) at genus level. (b) Weighted UniFrac clustering of 166,799 reads (representing 64.4% of all reads) based on a set of 258 sequences with total abundances between 16577 and 100. Capital letters indicate subclusters of infants according to common dominant bacterial genera in their microbiota. (A): *Bifidobacterium* spp. > 80%, (B): unclassified Lachnospiraceae 25 – 40%, (C): Enterobacteriaceae > 40%, and (D): *Streptococcus* >50%. Low risk (LR) individuals are shown in blue and high risk (HR) in red.

infants and three from high risk infants (HR12, HR13 and HR14) all containing 72 – 98% *Bifidobacterium* spp. grouped closely whereas three samples sharing the presence of 25 – 40% unclassified Lachnospiraceae (subcluster B) clustered together. Rarefaction curves for each group indicate that the total bacterial diversity was well represented since clustering curves for all individuals, except HR16, approached saturation at 90% (Data not shown).

### Richness and diversity analysis

Differences in richness and Simpson index between the high and low genetic risk groups of infants showed a borderline statistical significance ( $P = 0.065$ ) (Table 3). Richness was 18.1 [6.2] (mean [SD]) for the low genetic risk group and 27.0 [10.3] for the high genetic risk group. The respective Simpson indices were 0.68 [0.26] and 0.56 [0.23].

### Correlation analysis

The possible interactions between

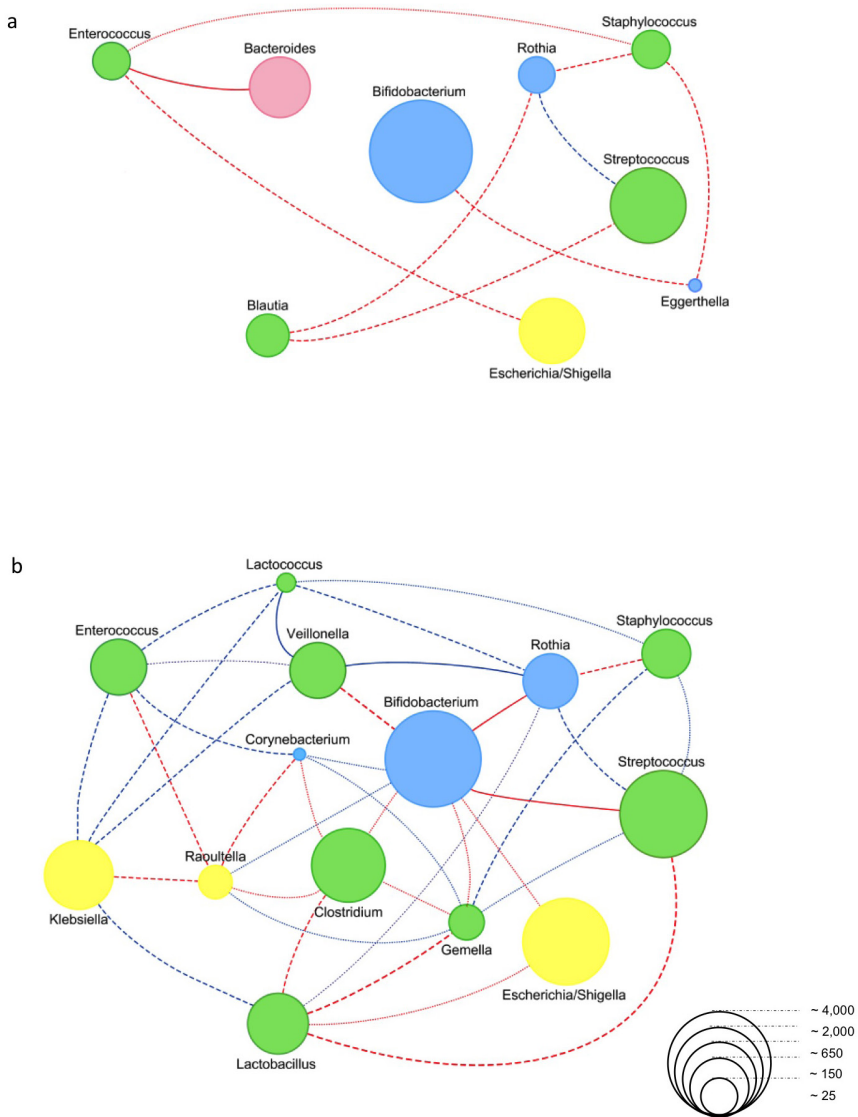
**Table 3:** Number of sequences obtained in samples from the low genetic risk (LR) and high genetic risk (HR) groups and percentage of reads assigned at the phylum, family and genus level.

Sample	No. of sequences	%assign. Phylum	%assign. Family	%assign. Genus	No. OTUs	Shannon index	Simpson index
LR1	8594	96.2	96.0	95.9	9	0.095	0.971
LR2	9513	98.5	98.5	98.3	13	0.104	0.962
LR3	10147	98.2	98.1	98.0	23	0.332	0.888
LR4	12248	99.6	99.6	99.6	10	0.252	0.909
LR5	9963	95.4	95.4	95.2	16	0.334	0.869
LR6	9770	93.6	93.5	93.4	21	0.886	0.475
LR7	9881	97.1	97.0	96.9	22	0.931	0.482
LR8	10628	96.2	95.9	95.6	22	1.215	0.343
LR9	7909	98.5	98.4	98.4	28	1.512	0.255
LR10	9896	99.7	99.7	99.7	13	0.540	0.775
LR11	12210	96.7	96.6	96.5	22	0.741	0.582
HR12	11568	92.7	86.9	85.3	8	0.152	0.947
HR13	13126	95.5	94.6	94.3	22	0.483	0.825
HR14	10267	95.9	95.7	95.4	21	0.710	0.657
HR15	10067	99.1	99.0	98.9	21	1.272	0.372
HR16	15726	88.7	88.2	87.1	42	1.300	0.360
HR17	17080	96.7	96.5	96.3	29	0.984	0.518
HR18	20010	97.3	97.1	97.1	16	0.399	0.795
HR19	17660	94.4	94.0	93.6	33	1.646	0.262
HR20	13284	99.4	99.3	99.3	31	1.225	0.320
HR21	13869	96.8	96.6	96.5	37	1.121	0.474
HR22	14732	98.4	98.3	98.1	37	0.810	0.624

OTU numbers and Shannon and Simpson indices were calculated at a clustering level of 90%.

the bacterial genera detected in infants at low genetic risk are summarized in **Figure 4a**. Nine interactions involving nine bacteria were selected that comprise only one positive correlation between *Rothia-Streptococcus* ( $r = 0.630$ ,  $1-P = 0.029$ ). The highest negative correlations were between *Bacteroides-Enterococcus* ( $r = -0.756$ ,  $P = 0.014$ ), and *Bifidobacterium-Eggerthella* ( $r = -0.613$ ,  $P = 0.024$ ). The increased diversity in the microbiota of high risk infants resulted in a higher

number of significant correlations (a total of 35) involving 14 different bacterial genera (**Figure 4b**). *Bifidobacterium* showed a significant negative correlation with *Veillonella* ( $r = -0.590$ ,  $P = 0.010$ ), *Rothia* ( $r = -0.800$ ,  $P = 0.004$ ), *Streptococcus* ( $r = -0.761$ ,  $P = 0.007$ ) and borderline significant with *Escherichial/Shigella* ( $r = -0.497$ ,  $P = 0.059$ ) and *Clostridium* ( $r = -0.397$ ,  $P = 0.121$ ). Of bacterial genera that enabled to discriminate between the two groups of infants, *Bifidobacterium*



**Figure 4:** Representation of the correlations among bacterial genera that met the criteria  $|1-P| < 0.100$  (positive correlation) or  $P < 0.100$  (negative correlations) in low (a) and high genetic risks groups (b) of infants. The color of the different genera indicate they belong to the phyla Actinobacteria (blue), Firmicutes (green), Proteobacteria (yellow) and Bacteroidetes (red). Circle sizes are relative to the logarithm of the mean weighted number of reads (see scale). Blue or red node colors indicate positive and negative correlations, respectively. Node style represents the correlation coefficient as continuous line ( $R \geq 0.75$  or  $\leq -0.75$ ), discontinuous line ( $|R| = 0.75 - 0.1$ ) and weak line ( $0.1 > R > -0.1$ )

showed a no significant positive correlation with *Corynebacterium* ( $r=0.103$ ,  $1-P=0.627$ ) and *Raoutella* ( $r=0.070$ ,  $1-P=0.582$ ) and negative with *Gemella* ( $r=-0.206$ ,  $P=0.270$ ). *Clostridium* spp. showed a statistically significant negative correlation with *Lactobacillus* ( $r=-0.573$ ,  $P=0.041$ ), but not with *Corynebacterium* ( $r=-0.130$ ,  $P=0.379$ ), *Gemella* ( $r=-0.212$ ,  $P=0.268$ ) and *Raoutella* ( $r=-0.057$ ,  $P=0.438$ ).

### **Prevalence of *Clostridium* sensu stricto discriminates between the infant groups**

The genus *Clostridium* sensu stricto was considered indicative of differences in the microbiota between low and high genetic risk groups, because its proportions were quantitatively different between both groups and it was present in nine individuals of the high genetic risk group but in none of the low genetic risk group. Therefore, the composition of this genus was analyzed in more detail.

*Clostridium* sensu stricto was present in three high risk group individuals representing 28.1 (HR20), 14.0 (HR21) and 13.3% (HR17) of all reads and in five more representing between 0.4 and 1.4%. The 10,173 reads classified in this genus were therefore further analyzed. In total, these represented 1,879 different sequence types. BlastN comparisons showed nine *Clostridium* species as best hits (Table 4). Three of them were dominant, to which

9,577 reads (94.1%) belonged to *Clostridium paraputrificum* (41.2%), *C. chartatabidum* (33.2%) and *C. perfringens* (19.8%) with average sequence similarities to the reference sequences X75907, X71850, and CP000246 of 97.8, 98.3 and 99.2%, respectively. *C. perfringens* was present in five individuals, proving the most widely distributed species. The highest diversity of *Clostridium* was found in individuals HR20 and HR21, with seven and five of the nine species identified, respectively. Interestingly, two closely related species (*C. paraputrificum* and *C. chartatabidum*) were coexisting in HR20, both in relatively high numbers. On the contrary, the presence of *C. perfringens* was practically exclusive.

### **Real time quantitative PCR (qPCR)**

The characterization of the microbiota composition by qPCR (Table 5) revealed that infants in the high genetic risk group had reduced gene copy numbers of *Bifidobacterium* spp. (median log values [interquartil] 6.86 [6.36-11.28] versus 11.00 [10.73-11.42]  $P=0.018$ ) compared to the low genetic risk group according to pyrosequencing data. The high genetic risk group also had reduced gene copy numbers of *Bacteroides fragilis* group and *Blautia* (*C. coccoides*) group compared to the low genetic risk group (> 1 log unit difference), but the latter differences did not reach statistical significance

**Table 4:** Distribution of pyrosequencing reads classified as *Clostridium* sensu stricto.

Best hit		Individual samples*									
Species	Acc. no.	All indiv.	HR 13	HR 14	HR 15	HR 16	HR 17	HR 19	HR 20	HR 21	HR 22
<i>C. paraputificium</i>	X75907	4190	2			5		60	4118	5	
<i>C. chartatabidum</i>	X71850	3376							1268	2108	
<i>C. perfringens</i>	CP000246	2011	203	84		81	1461				182
<i>C. vincentii</i>	X97432	306							79	227	
<i>C. isatidis</i>	X98395	140							42	98	
<i>C. sartagoforme</i>	Y18175	106							106		
<i>C. roseum</i>	Y18171	34							27	7	
<i>C. butyricum</i>	AJ458420	9			9						
<i>C. tertium</i>	Y18174	1							1		
	All types	10173	205	84	9	86	1461	60	5641	2445	182

Results of BlastN matches against reference sequences from the LTP-Silva database.

\*Samples HR12 and HR18 did not contain reads classified into this genus.

**Table 5:** Fecal microbiota of infants with high or low genetic risk of developing CD determined by qPCR.

Bacterial groups	Low genetic risk group	High genetic risk group	*P value
	log gene copy numbers/g stools		
	Median (IQR)		
<i>Bacteroides fragilis</i> group	7.62 (5.86-8.11)	6.04 (5.53-8.03)	0.336
<i>Bifidobacterium</i> spp.	11.00 (10.73-11.42)	6.86 (6.36-11.28)	<b>0.018</b>
<i>Lactobacillus</i> group	6.80 (5.15-9.45)	6.43 (5.28-9.69)	0.470
<i>Staphylococcus</i> spp.	6.81 (5.78-7.20)	8.41 (5.96-9.41)	0.142
<i>Enterococcus</i> spp.	8.15 (6.67-9.08)	8.26 (6.92-8.83)	0.922
<i>Streptococcus</i> spp.	8.90 (7.90-8.99)	8.98 (7.64-9.81)	0.563
<i>C. leptum</i> group	5.28 (4.12-6.53)	4.68 (4.13-8.92)	0.555
<i>Blautia</i> ** group	8.20 (5.42-10.13)	6.88 (6.02-10.34)	0.630

\*Statistically differences were established at  $P < 0.050$  applying the Mann-Whitney U test.

\*\* Formely *Clostridium coccooides* group.



( $P= 0.336$  and  $0.630$ , respectively). The high genetic risk group of infants also showed increased gene copy numbers of *Staphylococcus* spp. compared to the low genetic risk group; however, the difference was not statistically significant ( $P= 0.142$ ).

Statistically significant correlations between the data obtained by qPCR and pyrosequencing were detected for *Bacteroides fragilis* group ( $r= 0.998$ ,  $P< 0.001$ ), *Bifidobacterium* spp. ( $r= 0.469$ ,  $P= 0.037$ ) and *Lactobacillus* group ( $r= 0.869$ ,  $P< 0.001$ ). No significant correlation between the data obtained by the two techniques was detected for the other bacterial groups analyzed by qPCR.

## DISCUSSION

Emerging evidence supports the hypothesis that host genotype influences gut microbiota composition, but there is a limited number of studies revealing these interactions (33). The present study is the first to demonstrate by high-throughput sequencing of 16S rRNA genes that the HLA-DQ2 genotype *per se* influences the intestinal bacterial community structure of infants at family risk of developing CD. Indeed, compared to low risk infants, the intestinal microbiota of high risk infants was characterized by increased Firmicutes and Proteobacteria proportions but reduced Actinobacteria

proportions. The increased Firmicutes proportions were reflected by increased proportions of the genera *Clostridium* (*Clostridium sensu stricto* and unclassified Clostridiaceae) and *Gemella*. The higher abundances of Proteobacteria were due to increases in *Raoultella* and unclassified Enterobacteriaceae. The reduced proportions of Actinobacteria were due to decreases of the genera *Bifidobacterium* and *Corynebacterium*.

Reductions in *Bifidobacterium* spp. have also been detected previously in duodenal biopsies and feces of subjects with the disease, even after long-term adherence to a gluten-free diet, indicating that this bacterial group could contribute to the etiopathogenesis of CD (17, 18). Previous studies have investigated the influence of the HLA-DQ2/8 genotype on the intestinal microbiota composition of this cohort of infants with a family history of CD, but they used molecular techniques with a more limited resolution such as FISH, qPCR and denaturing gradient gel electrophoresis (22, 23, 34). Notwithstanding, initial results revealed that although both the HLA-DQ2/8 genotype and milk-feeding type (breast-milk or formula) influence the infant's gut colonization, infants with higher genetic risk (HLA-DQ2/8 positives) had lower *Bifidobacterium* spp. and *B. longum* numbers, and higher *Staphylococcus* spp. numbers irrespective of milk-feeding type

(23). However, our present study conducted in exclusively breast-fed infants did not reveal differences in *Bacteriodes* spp., confirming the hypothesis that breast-feeding attenuated the HLA-DQ-driven microbiota differences in this bacterial group, which were detected only in the formula-fed infants in our previous study by qPCR.

Our present pyrosequencing data also revealed that the HLA-DQ2 genotype influences colonization by *Clostridium* spp. (*Clostridium* sensu stricto and unclassified Clostridiaceae). *Clostridium* sensu stricto was detected in nine out of 11 samples from high risk infants, while it was absent in samples from low risk infants. *C. perfringens* was the species showing the highest prevalence (in five out of nine cases). A recent study that analyzed the intestinal microbiota by 16S rRNA gene pyrosequencing in children with the HLA-DQB1 (DQB1\*02, DQB1\*03:02) genotype,  $\beta$ -cell autoimmunity was positively associated with *C. perfringens* (35). However, this association could not be related to the HLA genotype because this was similar in children with  $\beta$ -cell autoimmunity (defined as positive diabetes-associated autoantibodies at least twice) and in those negative for the autoantibody. Also *Clostridium difficile* (cluster XI) infection, defined as positive toxin detection, has been associated with the NOD2 genotype and phenotype of patients with Crohn's disease (21).

In the present study, correlation analyses for high risk infants also revealed negative correlations between *Bifidobacterium* spp. and *Clostridium* spp., which might explain the absence of *Clostridium* spp. in low risk infants where *Bifidobacterium* spp. are clearly dominant. Data from a previous prospective study reported that a lower ratio of *Bifidobacterium* to *Clostridium* counts in the fecal microbiota of infants was associated with atopic disease (15).

Our pyrosequencing study has also been the first to detect differences in proportions of the genera *Corynebacterium*, *Gemella* and *Raoultella* as a function of the HLA-DQ2 genotype in the infant's gut. Although the biological role of these genera is not well-documented, mice with alcoholic liver disease also showed increases in *Corynebacterium* and *Alcaligenes* (36). The authors proposed that these changes in microbiota might contribute to the disease by increasing intestinal permeability via alterations in tight-junction related protein expression (zonula occludens-1 and claudin-1), which could be prevented by probiotic administration (36). In the light of our results, we cannot disregard the possibility that specific microbiota alterations (e.g. *Corynebacterium* spp.) may also contribute to this phenomenon in CD as increased intestinal permeability is also characteristic of the active phase of CD and is considered a potential pre-developmental event (37).

Another study analyzed the microbiota of a small subset of subjects (eight per group) by pyrosequencing to investigate how early (from 6 months on) or late (after 12 months) gluten introduction into the infant diet affected CD development in genetically predisposed infants (HLA-DQ2/8) (38). The authors reported that the microbiota of HLA-DQ2/8 carriers was characterized by higher abundance of Firmicutes and lower abundance of Bacteroidetes (1% to undetectable) as compared to data from another study on non-genotyped healthy infants (39). However, samples from each of the aforementioned studies were analyzed using different techniques (SSU rDNA microarray *versus* 454 pyrosequencing) which could explain the differences that the authors attributed to the HLA-DQ genotype (38). Differences in sample handling (storage time and temperature) and processing (e.g. DNA extraction method) could also lead to different results from massive sequencing analysis; therefore, studies using different methodologies are considered non cross-comparable (40). Furthermore, the genotype of infants included in the Palmer study was unknown (39), thus there may have been HLA-DQ carriers, because they represent up to 40% of the general population (5).

Another prospective study that analyzed possible associations between the intestinal microbiota composition and the risk of

developing neonatal sepsis or systemic inflammation revealed a dominance of Proteobacteria in infants who developed the disease (12) as in the case of our high genetic risk infants. Furthermore, correlation analyses in high risk infants also revealed negative correlations between *Bifidobacterium* spp. and Proteobacteria (*Escherichia/Shigella*), which might explain reduced levels of the latter in low risk infants whose microbiota was dominated by bifidobacteria. Also, the phenotype of patients with inflammatory bowel diseases (Crohn's disease and ulcerative colitis), and the NOD2 and ATG16L1T300A genotypes have been associated with compositional shifts in the phylum Proteobacteria in intestinal biopsies (41).

In the present study, statistically significant correlation between pyrosequencing and qPCR data was only obtained for bifidobacteria, bacteroides and lactobacilli. This can be partially explained by differences between the range of bacteria targeted by the qPCR primers used, and the classification of the corresponding taxa by pyrosequencing, as previously reported (40).

Several studies have attempted to establish relationships between gut microbiota diversity and health status, but conclusions have been rather inconsistent to date. It is well known that breast-fed babies have a low diverse microbiota, mainly dominated by the genus *Bifidobacterium*, and breast-feeding

exerts a positive effect over infants' health (42, 43). In our present study, infants with low genetic risk of developing CD also had lower bacterial diversity compared to high risk infants. Especially, in those high risk infants in which bifidobacteria were absent or present in only small numbers, the microbiota was much more diverse. Other studies report that decreased gut microbiota diversity is associated with diseases such as necrotizing enterocolitis (44) and type 1 diabetes (45) in infants, and with obesity in adults (46). A prospective study of neonatal infants also showed that those who developed sepsis or systemic inflammation presented low microbial diversity, from the meconium through to disease onset (12). One of the reasons that can explain the controversial conclusions regarding the relationship between microbial diversity and disease reported in infants is that some of the previous studies have used primers that are not appropriate for detection of bifidobacteria (12, 44, 45), which is the bacterial group that mainly determine gut microbiota diversity early in life. Underrepresentation of bifidobacteria in 16S rRNA pyrosequencing analyses has been described and led to the design of alternative wobbled PCR amplification primers that improve the detection of bifidobacteria (47) and that were used in our study. In conclusion, the results of our study based on high-throughput

sequencing reveal that the HLA-DQ2 genotype strongly influences the intestinal colonization of infants at family risk of developing CD. Infants with high genetic risk of developing the disease (HLA-DQ2 carriers) show reduced abundance of Actinobacteria (*Bifidobacterium* spp.) and increased abundance of Firmicutes (*Clostridium* sensu stricto and unclassified Clostridiaceae and *Gemella*) and Proteobacteria (*Raoultella* and unclassified Enterobacteriaceae). Some of these bacterial groups were negatively correlated, suggesting that one excludes the others. Follow-up of this study cohort is underway to determine whether the microbiota changes dictated by the HLA-DQ genotype precede the development of CD in later life.

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**SUPPLEMENTARY TABLE**

**Sup. table 1:** List of haplotypes and their respective DQA1 and DQB1 genotype.

Haplotype	HLA genotype
DQ2	DQB1*02
DQ2.2	DQA1*02:01 DQB1*02:02
DQ2.5	DQA1*05:01 DQB1*02:01
DQ4	DQB1*04
DQ4.2	DQA1*04:01 DQB1*04:02
DQ5	DQA1*01 DQB1*05
DQ5.1	DQA1*01:01 DQB1*05:01
DQ5.3	DQA1*01:04 DQB1*05:03
DQ6	DQA1*01 DQB1*06
DQ6.1	DQA1*01:03 DQB1*06:01
DQ7	DQA1*05 DQB1*03:01
DQ7.5	DQA1*05:05 DQB1*03:01
DQ8	DQA1*03:01 DQB1*03:02



## Chapter 4

- **Double-blind, randomized, placebo-controlled intervention trial to evaluate the effects of *Bifidobacterium longum* CECT 7347 on children with newly diagnosed celiac disease**



# **Double-blind, randomized, placebo-controlled intervention trial to evaluate the effects of *Bifidobacterium longum* CECT 7347 on children with newly diagnosed celiac disease**

Marta Olivares<sup>1</sup>, Gemma Castillejo<sup>2</sup>, Vicente Varea<sup>3</sup>, and Yolanda Sanz<sup>1</sup>

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<sup>1</sup>Microbial Ecology, Nutrition & Health. Institute of Agrochemistry and Food Technology. National Research Council (IATA-CSIC). Valencia, Spain. <sup>2</sup>Paediatric Gastroenterology Unit, Hospital Universitari Sant Joan de Reus, Universitat Rovira i Virgili. Tarragona, Spain. <sup>3</sup>Paediatric Gastroenterology, Nutrition and Hepatology. Hospital Universitario Sant Joan de Deu. Barcelona, Spain.



## ABSTRACT

Interactions between immune system and intestinal microbiota may play a role in celiac disease (CD). The potential effects of *Bifidobacterium longum* CECT 7347 on children with newly diagnosed CD were evaluated. A double-blind, randomized, placebo-controlled trial was conducted in 33 children that received a capsule containing either *B. longum* CECT7347 ( $10^9$  colony-forming units) or placebo (excipients) daily for 3 months in parallel to the gluten-free diet. Outcome measures (baseline and after intervention) included immune phenotype of peripheral-blood cells, serum cytokines, faecal secretory immunoglobulin A (sIgA), anthropometric parameters and intestinal microbiota. Comparisons between groups revealed greater height percentile increases ( $P= 0.048$ ) in the *B. longum* CECT 7347 group than in the placebo group, as well as decreased peripheral CD3<sup>+</sup> T-lymphocytes ( $P= 0.004$ ) and slightly reduced tumour necrosis factor (TNF)- $\alpha$  concentration ( $P= 0.067$ ). Within group comparisons of baseline and final values did not reveal differences in T-lymphocytes and cytokines in the placebo group, while decreased CD3<sup>+</sup> ( $P= 0.013$ ) and HLA-DR<sup>+</sup> T lymphocytes ( $P= 0.029$ ) and slightly reduced TNF- $\alpha$  concentration ( $P= 0.085$ ) were detected in the *B. longum* CECT 7347 group. Comparison between groups showed that *B. longum* CECT 7347 administration reduced *Bacteroides fragilis* group numbers ( $P= 0.020$ ) and stool sIgA ( $P= 0.011$ ) in comparison with the placebo. Although this is a first exploratory intervention with limitations, the findings suggest that *B. longum* CECT 7347 could help to improve the health status of CD patients, who tend to show alterations in gut microbiota composition and a biased-immune response even on a GFD.

**Keywords:** celiac disease, *Bifidobacterium longum* CECT 7347, probiotics, gluten-free diet.

## INTRODUCTION

Celiac disease (CD) is an autoimmune enteropathy caused by a permanent intolerance to dietary proteins of wheat, barley and rye in genetically predisposed (human leukocyte antigen (HLA)-DQ2/8) individuals. In CD patients, gluten proteins induce a deregulated immune response that typically leads to severe small intestine mucosal injury, malabsorption and gastrointestinal symptoms (1). Gluten proteins are the main environmental factor involved in CD pathogenesis; however, recent studies have shown that other factors influencing the interplay between the gut microbiota and the mucosal immune system, including breastfeeding and gastrointestinal infections, could contribute to determining the disease onset (2). Observational studies conducted so far have reported associations between intestinal dysbiosis and celiac disease (3), with a few exceptions (4, 5). Our own studies reported that the duodenal and faecal microbiota of CD patients is unbalanced with decreased numbers of *Bifidobacterium* spp. and increased numbers of *Bacteroides* spp. and *E. coli* clones with higher virulence features, which are only partially normalized after a long-term gluten-free diet (GFD) (6-8). One of the most recent studies has also reported that CD patients with gastrointestinal symptoms had different microbiota

composition in comparison with controls and patients with dermatitis herpetiformis, suggesting that the microbiota may play a role in the manifestation of the disease (9). *In vitro* and animal studies have demonstrated that the dysbiotic gut microbiota of CD patients and specific isolates may potentially enhance the inflammatory response elicited by gluten and contribute to increasing intestinal epithelial permeability and gliadin peptide translocation (10-13). In contrast, *Bifidobacterium longum* CECT 7347 has been shown to attenuate the inflammatory effects of the dysbiotic gut microbiota of CD patients on peripheral blood mononuclear cells (PBMCs) partially via induction of interleukin (IL)-10 production (10, 14). This strain has also been proven to reduce the cytotoxic and inflammatory effects of gliadin peptides on epithelial cells *in vitro* via degradation of gliadin peptides (15, 16). *B. longum* CECT 7347 administration has also been shown to reduce the numbers of peripheral T CD4+ cells, and increase IL-10 and reduce tumour necrosis factor (TNF)- $\alpha$  production in jejunal sections in an animal model of gliadin-induced enteropathy (17). The only current treatment for CD is adherence to a strict GFD, which usually results in symptomatic, serologic and histological remission (18, 19). However, compliance with a strict GFD is very difficult due to the presence of gluten in

most processed foods and the social restriction implied; thus overall the GFD is far from optimal. In practice, CD patients may continue suffering from clinical symptoms, nutritional deficiencies and higher health risks (20-22). For example, it has been reported that even under GFD, CD children (23) and women (24) have a much higher prevalence of gastrointestinal symptoms than do controls, and they also use healthcare services more often (22). Whether or not this is due to residual chronic inflammation, exposure to minimum amounts of gluten and/or intestinal dysbiosis remains unknown. Whether complete normalization of lymphocyte subsets in peripheral blood can be achieved remains unclear (19, 25). Other studies also indicate that small intestine bacteria overgrowth is often associated with non-responsive CD (26). The duodenal and faecal microbiota of CD patients has also been shown not to be completely normalized after adherence to a GFD, which can also contribute to the lack of complete restoration of the intestinal immune homeostasis in the patients (6, 7). Therefore, adjunctive strategies that can improve the effects of a GFD are being investigated (27).

The aim of this study was to conduct a three-month double-blind randomized placebo-controlled trial on children with newly diagnosed CD following a GFD in order to evaluate the effects of *B. longum* CECT 7347 administration on

immune and anthropometric parameters, and on the intestinal microbiota composition.

## MATERIAL AND METHODS

### Subjects and study design

A total of 36 children were recruited at the Hospital Universitari Sant Joan (Reus, Tarragona) and Hospital Universitario Sant Joan de Deu (Barcelona) from November 2011 to June 2012. The inclusion criteria were children aged 2-17 years with newly diagnosed CD according to the European Society of Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) criteria, based on symptoms, positive anti-tissue transglutaminase antibodies quantified using tTG-IgA ELISA kit (Pharmacia Diagnostics GmbH, Freiburg, Germany) and duodenal biopsy demonstrating villous atrophy. The exclusion criteria did not allow subjects enrolled in another clinic study, treated with antibiotics 30 days before starting the study, and/or with food allergy or others pathologies. Demographic and clinical characteristics of the subjects included in the study are shown in **Table 1**.

A double-blind, randomized, placebo-controlled intervention trial was conducted. A graphic representation of the study protocol is shown in **Figure 1**. All children recruited (n=36) met the inclusion criteria and were randomly assigned

**Table 1.** Clinical characteristics of the subjects of the two study groups

Characteristics	Study group		* <i>P</i> value
	Placebo	<i>B. longum</i> CECT 7347	
Number of cases	16	17	
Male/Female	8/8	7/10	0.437
Age, [mean (SE)]	8.5 (1.2)	6.8 (0.9)	0.265
Baseline height (cm) [mean (SE)]	26.5 (3.8)	21.8 (3.4)	0.301
Baseline weight (kg) [mean (SE)]	119.8 (5.7)	109.3 (6.4)	0.449
Height percentile baseline/post-treatment			0.530/0.530
Children up percentile 50	2/2	3/3	
Children at percentile 50	0/0	0/0	
Children under percentile 50	14/14	14/14	
Increased height percentile			
Mean (SE)	7.0 (1.5)	7.8 (2.2)	0.591
Post-treatment-baseline/baseline [median (IQR)]	0.30 (0.1-0.53)	1.29 (0.22-2.85)	0.048*
Weight percentile baseline/post-treatment			0.530/0.365
Children up percentile 50	3/3	4/6	
Children at percentile 50	0/1	0/0	
Children under percentile 50	13/12	13/11	
Increased weight percentile			
Mean (SE)	3.2 (1.7)	5.7 (1.8)	0.186
Post-treatment-baseline/baseline [median (IQR)]	0.22 (0.09-0.47)	0.26 (0.14-1.64)	0.234
Histology (Marsh categorization)**			0.701
Marsh 1	0	1	
Marsh 2	0	1	
Marsh 3a	5	3	
Marsh 3b	8	10	
Marsh 3c	3	2	
Anti-transglutaminase antibodies (post-treatment-baseline/baseline) [median (IQR)]	-0.60 (-0.88 to -0.61)	-0.84 (-0.88 to -0.75)	0.418

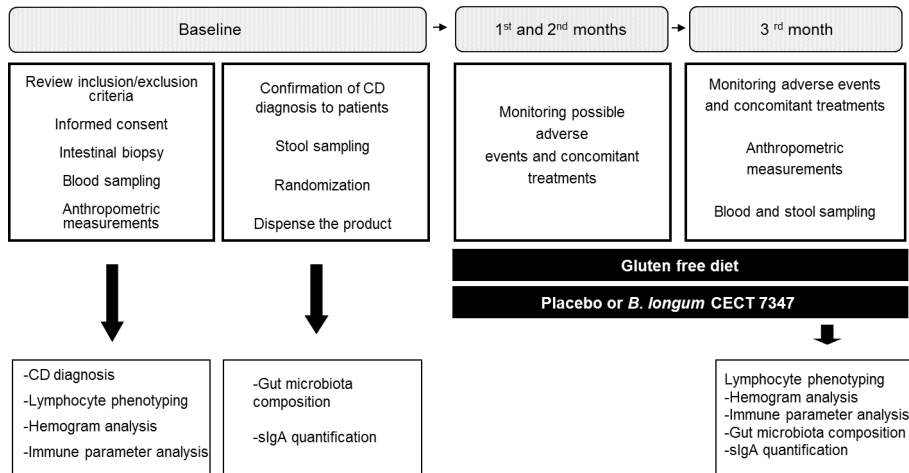
\*Categorical variables were analysed using the Fisher test and continuous variables were analysed using the t-test except for few exceptions for which the Mann-Whitney *U* test was applied (increased height and weight percentile expressed as quotient of post-treatment-baseline/baseline and changes in the anti-transglutaminase antibodies content) for comparison between groups (placebo *versus B. longum* CECT 7347). In all the cases differences were established at a *P* value < 0.050.

\*\* Histological data were obtained only at the diagnosis stage.

to the *B. longum* CECT 7347 (n=18) or the placebo (n=18) group. The randomization design was generated using the SAS module PROC PLAN (9.1.3 version, service pack 2) multiple of two sized blocks and 1:1 pattern through a centralized randomization list. The test group received *B. longum* CECT 7347

(109 CFU per capsule daily) and the placebo group received an indistinguishable capsule containing only the excipient (skimmed milk with 30% sucrose and 0.5% vitamin C daily) taken at lunch time or with other food intake, for 3 months after CD diagnosis and in parallel to the GFD. Capsule groups were labelled





**Figure 1:** Graphical representation of the study protocol and the timing of sample collection and assessments during the 3 months of intervention.

with codes consisting of a letter and 3 numbers assigned randomly by an external scientist involved in bacterial strain production but not in its evaluation in the present study. Study personnel, including clinicians and research assistants involved in experimental work and data analysis, were *naïve* as to which treatment these labels represented. All these measures ensured true allocation concealment and proper blinding. Visits to paediatricians and parameters monitored are shown in **Figure 1**. The trial was not registered because it was a first exploratory study.

### Gluten free diet (GFD) and dietary records

Children adhered to a strict GFD, after confirmation of CD diagnosis, by replacing gluten-containing foods by equivalent ones certified as

gluten free (less than 20 ppm [mg/kg] according to the EC Regulation N°41/2009). Dieticians provide dietary counselling to select naturally and certified gluten-free products. They were also advised not to consume prebiotic and probiotic containing foods. To control diet as variable, food diary records were collected for 72 h (2 weekdays and 1 weekend day) before the start of the intervention and at the end of the 3 months of intervention. At the front of the diary, detailed information on how to record food and beverages consumed using common household measure was provided. Food diary records were returned to the dietician for their analysis for energy, macronutrient and micronutrients contents based on the CESNID food composition database of Spanish foods (28).

### **Clinical assessments**

At baseline and after the three-month intervention, serologic markers of CD, anthropometric parameters (height and weight) and evolution of clinical symptoms were assessed by paediatricians specialized in gastroenterology. Parents were also instructed to weekly register data on compliance with probiotic intake, gastrointestinal symptoms (presence or absence of vomiting, diarrhoea, constipation or abdominal pain), and concomitant treatments (e.g. antibiotics, drugs interfering with intestinal function, etc.) and serious adverse events (hospitalization, any life-threatening event, etc.) and to report them to the paediatrician at each visit. Compliance was considered to be accomplished when the intake of the test capsules was not interrupted for more than two consecutive days and no more than three times during the whole intervention period (three months). Parents were also provided a round-the-clock call service and e-mail address for immediate reporting if necessary.

### **Lymphocyte phenotyping**

At baseline and the end of the three-month trial period, peripheral blood samples were collected in heparin tubes and separated in two aliquots; one was mixed with stabilizing blood Transfix reagent (Cytomark, Buckingham, UK) and sent immediately to IATA-CSIC at room temperature for cytometry

analysis. The second blood sample was centrifuged (1,200 xg, 10 min, 4 °C) and the serum was stored at -80°C. Furthermore, a routine hemogram was carried out with all the blood samples.

Flow cytometry was used to determine the expression of surface markers of different lymphocyte subsets. Aliquots (100 µL) of stabilized peripheral blood were incubated with different fluorochrome-conjugated antibodies (eBiosciences, Hattfield, UK) at room temperature for 10 minutes and then prepared for flow cytometry analysis using the Immunoprep kit (Beckman Coulter, USA) according to the manufacturer's instructions. Samples were analysed in a FACS-Canto II Deckton Dickinson cytometer (BD Bioscience). To analyse the T lymphocytes subsets cells were stained with the following monoclonal antibodies: CD45-phycoerythrin (PE) (clone HI30), CD3-allophycocyanin (APC) (clone OKT3), HLA-DR-fluorescein isothiocyanate (FITC) (clone LN3), CD4-FITC (clone OKT4), Foxp3-APC (clone PCH101) and CD8-APC (clone RPA-T8). Lymphocyte subsets were classified based on the pattern of their surface marker expression as CD45+CD3+ (mature T lymphocytes); CD45+HLA-DR+ (late activation marker positive T lymphocytes); CD45+CD4+ (helper T cells); CD45+CD4+ and Foxp3+ (regulatory T cells) and CD45+CD8+ (cytotoxic T

cells). Data from 100,000 events were acquired and percentages of each subpopulation related to the gated lymphocytes labelled with the leukocyte marker CD45+ were estimated using the BD FACSDiva™ software v5.0.

### **Cytokine and immunoglobulin quantification**

In serum samples, the initial and final content of the pro- and anti-inflammatory cytokines (TNF- $\alpha$ , interferon (IFN)- $\gamma$ , IL-13 and IL-10) were quantified using a commercial ELISA kit following the manufacturer's instructions (Catalogue n° 31673019, 31333539 and 31330139, Immunotools, Friesoythe, Germany; Catalogue n° 430605, Biolegend, Cambridge, UK). Transforming growth factor (TGF)- $\beta$ 1 analysis included the activation of the molecule (incubation with 1 N HCl for 1 hour and neutralization with 1 N NaOH) prior to its quantification with an ELISA (Catalogue n° 437708, Biolegend, Cambridge, UK). Immunoglobulin (Ig) G1 (Catalogue n° ELH-IGG1-001, RayBiotech, Gwinnett, USA) and IgG4 (Catalogue n° CSB-E13728h, Cusabio, Wuhan, China) content were also quantified using specific ELISA kits.

### **Secretory IgA (sIgA) quantification**

Faecal samples were collected at baseline and at the end of the trial and stored at -80 °C. One gram

of faecal sample was weighed, diluted 1/10 (w/v) with PBS buffer (130 mM sodium chloride and 10 mM sodium phosphate buffered saline, pH 7.4) and homogenized in a stomacher for 5 minutes. The mixture was also homogenized in a tube with glass beads of 5 mm using a vortex and subjected to low-speed centrifugation (600  $\times$ g, 2 min). The supernatants were collected for sIgA quantification by ELISA according to the manufacturer's instructions (Catalogue n° E80-102, Bethyl, Montgomery, USA) as well as for DNA extraction as described below.

### **Microbiota analysis by real-time PCR**

DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Real-time PCR analyses were run to quantify the content of different bacterial groups and *Bifidobacterium* spp. using specific primers as previously described (Supplementary table 1). PCR amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in a multi well-plate with 15  $\mu$ L of reaction mixture that consisted of 7.5  $\mu$ L SYBR Green PCR Master Mix (Roche), 3.5  $\mu$ L DNase free water, 0.75  $\mu$ L of each specific primer (10 mM) and 2.5  $\mu$ L of the DNA sample. 16S rRNA gene copy numbers were calculated by comparing the cycle threshold (Ct) values obtained with those from a

standard curve (29). Standard curves were generated from serial dilutions of a known copy number of the target gene cloned into a plasmid vector. For each reference strain the 16S rRNA gene was cloned into a pGEM-T Easy Vector System (Promega, Madison, USA) and an *E. coli* DH5 $\alpha$  strain was transformed with the recombinant plasmids. Plasmid DNA was extracted from *E. coli* by the miniprep method (30).

### Statistical analysis

Data distribution of continuous variables was tested using Shapiro-Wilk  $W$  test. When data showed a normal distribution ( $P > 0.050$ ) a  $t$ -test for paired sample comparisons was used and results are expressed as mean values and the standard error of the mean [SE]). When no-normal distribution was found, the Mann-Whitney  $U$  test was applied for paired sample comparisons and data are expressed as median and interquartile range (IQR). Microbiological data were transformed from exponential numbers into logarithms to adjust to normal. All parameters measured during the intervention were expressed as the difference between the baseline value and the post-intervention value, divided by the baseline value for pair comparisons between the two study groups applying either the  $t$ -test or the Mann-Whitney  $U$  test. A generalized linear model was also applied to absolute values of all parameters measured to establish differences between the two

study groups and possible time by treatment interactions. Correlations between the microbiota composition data and immunological data were stabilised by applying the Pearson coefficient.

Categorical demographic data of the study subjects (**Table 1**) and the incidence of gastrointestinal symptoms (**Table 2**) are expressed as absolute values or proportions and analysed using the Fisher test.

The analyses were conducted using the SPSS software (version 19.0, Inc, Chicago, IL, USA) and in all the cases, statistically significant differences were considered at a  $P$  value below 0.050.

### Ethical considerations

The study was approved by the ethics committees of Consejo Superior de Investigaciones Científicas (CSIC) and the hospitals involved in the study, Hospital Universitario Sant Joan (Reus, Tarragona) and Hospital Universitario Sant Joan de Deu (Barcelona) and conducted in accordance with the Helsinki Declaration of 1975 as revised in 1983. Written informed consent was obtained from the parents of children included in the study.

## RESULTS

### Trial subject characteristics

From November 2011 to June 2012 a group of 36 children newly diagnosed with CD were enrolled

in a randomized, double-blind, placebo-controlled intervention trial to evaluate the effects of intervention in the gut ecosystem with *B. longum* CECT 7347 in parallel to those of the GFD. Three withdrawals were registered one due to parents' complaints of the child's reduced appetite and two due to the intake of antibiotics. Two of the withdrawals belonged to the placebo treatment and one to the *B. longum* group. Finally, data from 17 subjects of the probiotic group and 16 of the placebo group were included in the statistical analysis. Taking account the number of the children enrolled, the mean missed doses in the placebo group was 1.3 and in the probiotic 1.9, which did not differ significantly ( $P=0.193$ ).

Dietary records did not revealed significant differences between groups in the intake of energy and specific nutrients ( $P> 0.050$ ). The adherence to the GFD only led to a slight reduction in the dietary fibre intake in both group of children (Supplementary table 2). Intake of other probiotics or prebiotics was not reported during the study period. The demographic and clinical characteristics of the child groups are shown in **Table 1**. Proper randomization was achieved as there were no significant differences in the baseline characteristics between the two groups. The serum antibodies to tissue transglutaminase (tTG)-IgA were normalized, decreasing to similar levels ( $P= 0.418$ ) in both

groups after 3 months of adherence to a GFD and intervention (**Table 1**). No adverse events were reported during the intervention. Height and weight data were analysed according to the WHO growth chart percentiles. As is shown in **Table 1**, at the time of diagnosis 84.8% (28/33) and 78.8% (26/33) of the children enrolled in the study were under percentile 50 (P50) for height and weight, respectively, corresponding to their age. After 3 months of intervention, two children in the *B. longum* CECT 7347 group and one child in the placebo group gained weight so as they were included in the group of children up P50 and in the group of children at P50, respectively. The mean increases in height and weight percentiles (difference in the percentile from baseline and post-treatment) were higher in the *B. longum* CECT 7347 group ( $\Delta 7.8$  [2.2] and  $\Delta 5.7$  [1.8], respectively) than in the placebo group ( $\Delta 7.0$  [1.5] and  $\Delta 3.2$  [1.7], respectively). Comparison of these changes between groups revealed that increases in height percentile were significantly greater in the *B. longum* CECT 7347 group than in the placebo group ( $P= 0.048$ ), while statistically significant differences in weight percentile increases were not detected ( $P= 0.234$ ).

The incidence of gastrointestinal symptoms (diarrhoea, constipation, abdominal pain and vomiting) in the two study groups during the three months of intervention are showed

in **Table 2**. No statically differences in the number of cases self-reporting incidence of these symptoms were detected either between groups or within the placebo or *B. longum* group.

### Lymphocyte phenotype and hemogram

The results of immune phenotyping of peripheral blood cells by flow cytometry are shown in **Table 3** and **Figure 2**. Within group comparisons did not reveal statistically significant changes in the placebo group during the three-month intervention on comparing baseline with post-treatment values (**Table 3**). In contrast, *B. longum* CECT 7347 administration led to significant decreases in CD3+ ( $P= 0.013$ ) and HLA-DR+ ( $P= 0.029$ ) T cell populations on comparing baseline with post-treatment values within this group (**Table 3**). Baseline values of the measured parameters were

not significantly different between groups. Comparisons between groups revealed that the administration of *B. longum* CECT 7347 significantly reduced ( $P= 0.004$ ) CD3+ T cell population as compared to the placebo (**Figure 2**). No statistically significant differences detected in the T lymphocytes subsets HLA-DR+, CD4+, CD4+Foxp3+ and CD8+ between groups ( $P= 0.328, 0.970, 0.504$  and  $0.376$ , respectively) (**Figure 2**).

Similar differences were detected between groups by applying a generalized linear model. This model also revealed a significant effect of the *B. longum* CECT 7347 intervention, consisting in a reduction of the CD3+ population that was opposite to the effect of the GFD ( $P= 0.020$ ) and a statistically significant time by treatment interaction ( $P= 0.021$ ). Baseline values for CD3+ were no different between the two groups ( $P= 0.950$ ).

**Table 2.** Number of cases self-reporting gastrointestinal symptoms at least once during the three-month intervention period in parallel to the GFD.

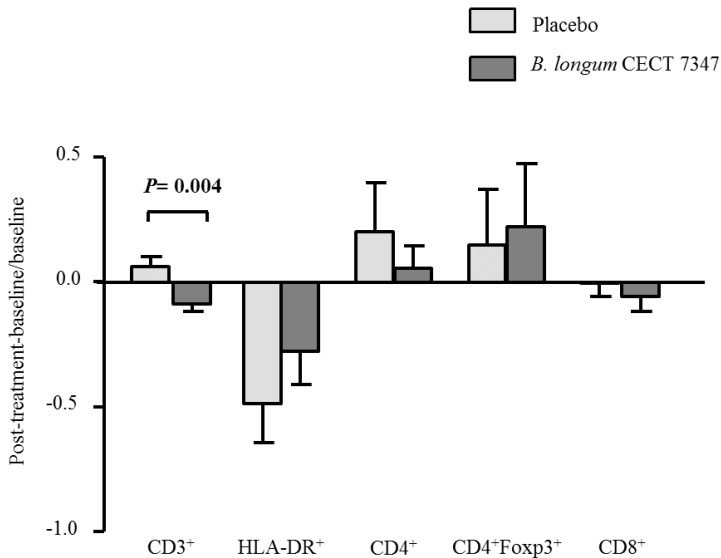
	N° of cases reporting gastrointestinal symptoms					
	Placebo (n=16)		*P-value	<i>B. longum</i> CECT 7347 (n=17)		*P-value
	Initial	Final		Initial	Final	
Diarrhoea	6/16	4/16	0.352	6/17	5/17	0.500
Constipation	3/16	2/16	0.500	3/17	2/17	0.500
Abdominal pain	6/16	4/16	0.352	10/17	9/17	0.500
Vomiting	5/16	1/16	0.087	4/17	1/17	0.206

\*Data were compared applying the Fisher test and differences were established at  $P$  value  $<0.05$ .

**Table 3.** Changes in CD45<sup>+</sup> T lymphocytes subsets within the placebo group or *B. longum* CECT 7347 group in the three-month intervention parallel to the GFD.

	Baseline		Post-treatment		* <i>P</i> value
	Mean	SE	Mean	SE	
<b>Placebo</b>					
CD3 <sup>+</sup>	66.96	2.85	70.03	2.17	0.144
HLA-DR <sup>+</sup>	0.99	0.19	0.72	0.24	0.422
CD4 <sup>+</sup>	41.78	4.37	42.60	4.89	0.898
CD4 <sup>+</sup> Foxp3 <sup>+</sup>	2.99	0.82	3.77	1.36	0.322
CD8 <sup>+</sup>	22.08	1.95	20.68	1.13	0.344
<b><i>B. longum</i> CECT 7347</b>					
CD3 <sup>+</sup>	70.05	2.23	63.42	2.04	0.013
HLA-DR <sup>+</sup>	0.62	0.10	0.38	0.82	0.029
CD4 <sup>+</sup>	44.68	2.40	45.96	3.59	0.748
CD4 <sup>+</sup> Foxp3 <sup>+</sup>	3.13	0.66	3.45	0.73	0.728
CD8 <sup>+</sup>	23.92	1.55	21.59	0.98	0.152

\*Differences between baseline and post-treatment within the study groups (placebo or *B. longum* CECT 7347) were established at two-sided  $P < 0.05$  using the t-test.



**Figure 2.** Changes in CD45<sup>+</sup> T lymphocytes subsets estimated as the difference between the baseline value and the post-intervention value, divided by the baseline value in placebo (light grey colour) and *B. longum* CECT 7347 (dark grey colour) groups in the three-month intervention parallel to the GFD. Data are expressed as mean and SE. Statistically significant differences between groups were established at two-sided  $P < 0.05$  using the Mann-Whitney *U* test.

No changes in the total leukocyte and leukocyte subtype (lymphocytes, monocytes, granulocytes, eosinophils and basophils) counts or haemoglobin content were observed on analysing the hemogram (**data not shown**), either within groups or between groups.

### Cytokines and immunoglobulin content

The results of serum cytokines and immunoglobulin concentrations are shown in **Table 4** and **Figure 3**. Within group comparisons, revealed that *B. longum* CECT 7347 administration slightly reduced

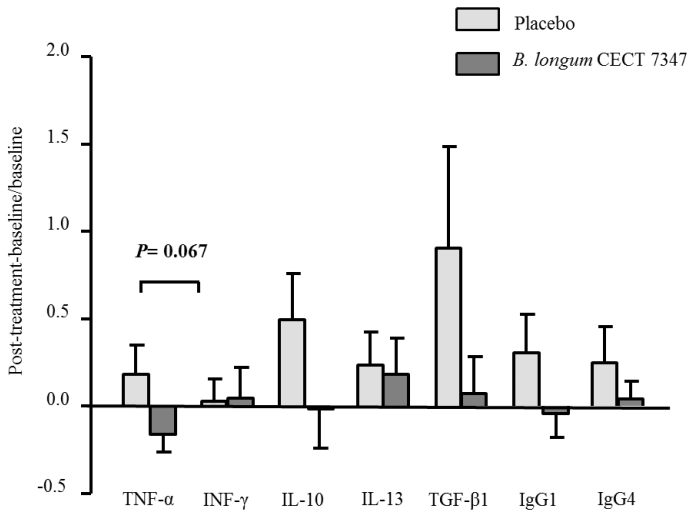
serum TNF- $\alpha$  concentrations, but differences did not reached statistical significance ( $P=0.085$ ). Changes in other serum cytokines and immunoglobulin concentrations, comparing baseline and post-treatment values within groups, were not significantly different in the *B. longum* CECT 7347 group or in the placebo group (**Table 4**). Comparisons between groups (*B. longum* CECT 7347 and placebo) of changes in serum cytokines and immunoglobulin levels are shown in **Figure 3**. Baseline values of the measured parameters were not significantly different between

**Table 4:** Changes in the cytokine (pg/mL) and immunoglobulin (ng/mL) content within the group placebo (a) or *B. longum* CECT 7347 (b) in the three-month intervention parallel to the GFD.

	Baseline		Post-treatment		* <i>P</i> value
	Mean	SE	Mean	SE	
Placebo					
TNF- $\alpha$	1669.56	338.47	1515.24	254.34	0.443
IFN- $\gamma$	306.42	32.59	297.59	36.32	0.815
IL-10	42.99	9.25	59.30	9.33	0.249
IL-13	137.71	12.85	163.86	21.16	0.239
TGF- $\beta$ 1	116.43	38.62	146.63	25.71	0.537
IgG1	441.85	84.04	477.39	94.43	0.644
IgG4	4.63	0.42	6.29	1.18	0.212
<i>B. longum</i> CECT 7347					
TNF- $\alpha$	1803.29	187.95	1442.94	153.20	0.085
IFN- $\gamma$	267.02	33.52	235.49	23.97	0.423
IL-10	69.14	12.15	52.02	5.28	0.240
IL-13	116.21	20.85	119.96	7.11	0.854
TGF- $\beta$ 1	130.67	34.41	138.42	33.52	0.851
IgG1	368.91	57.51	400.82	74.43	0.590
IgG4	4.38	0.19	4.42	0.34	0.919

\*Differences between baseline and post-treatment values within the group placebo or *B. longum* CECT 7347 were stabilised at two-sided  $P<0.05$  using the t-test.





**Figure 3.** Changes in the cytokine (pg/mL) and immunoglobulin (ng/mL) content estimated as the difference between the baseline value and the post-intervention value, divided by the baseline value in placebo (light grey colour) and *B. longum* CECT 7347 (dark grey colour) groups in the three-month intervention parallel to the GFD. Data are expressed as mean and SE. Statistically significant differences between groups were established at two-sided  $P < 0.05$  using the t-test.

groups. The administration of *B. longum* CECT 7347 slightly reduced serum TNF- $\alpha$  concentration compared to the placebo but statistical significance was not reached ( $P = 0.067$ ). No statistically significant differences were detected in serum IL-10, IFN- $\gamma$ , IL-13, TGF- $\beta$ 1, IgG1 and IgG4 concentrations between groups.

Applying a generalized linear model significant differences in IgG4 ( $P = 0.038$ ) were detected, showing increased values in the placebo group. Significant effects or interactions were not detected for other parameters.

### Microbiota composition and faecal sIgA content

Within group changes in microbiota composition as a result of the interventions showed that the GFD plus placebo administration caused a statistically significant increase in gene copy numbers of the *Bacteroides fragilis* group ( $P = 0.013$ ) and in the family *Enterobacteriaceae* ( $P = 0.038$ ) (Table 5). *Bifidobacterim* spp. gene copy numbers were also slightly reduced as a consequence of the GFD although differences did not reach statistical significance ( $P = 0.151$ ). Within group comparisons also showed that the ratio of *Lactobacillus* group + *Bifidobacterium* spp./*Bacteroides fragilis* group + *Enterobacteriaceae* was significantly

**Table 5.** Changes in intestinal microbiota composition (log of gene copy numbers/g stools) within the group placebo or *B. longum* CECT 7347 in the three-month intervention parallel to the GFD.

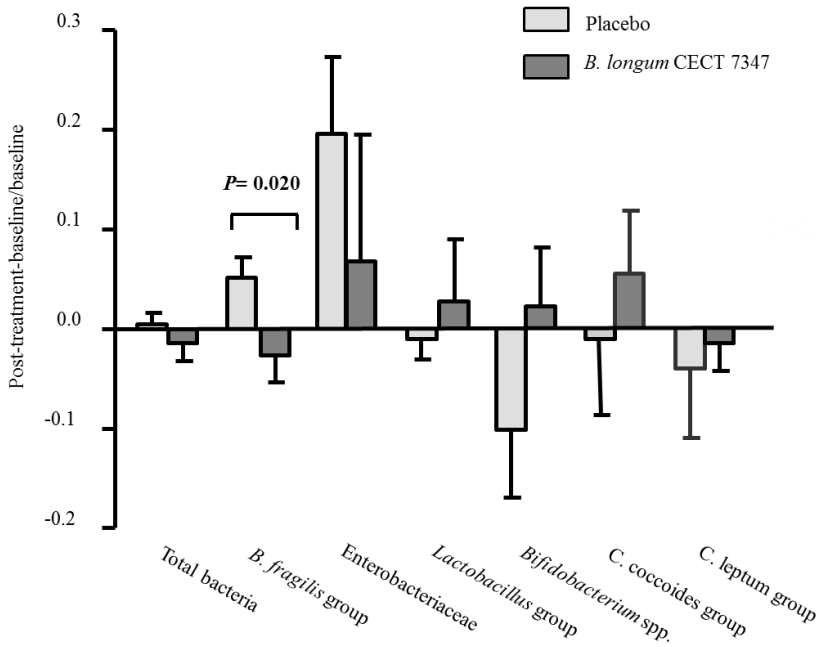
	Baseline		Post-treatment		<i>P</i> value
	Mean	SE	Mean	SE	
<b>Placebo</b>					
Total bacteria	11.10	0.14	11.15	0.18	0.689
<i>B. fragilis</i> group	9.07	0.17	9.59	0.22	0.013
<i>Enterobacteriaceae</i>	6.03	0.38	6.93	0.34	0.038
<i>Lactobacillus</i> group	8.55	0.14	8.45	0.18	0.589
<i>Bifidobacterium</i> spp.	9.37	0.19	9.01	0.24	0.151
<i>C. coccoides</i> group	8.85	0.33	9.31	0.28	0.115
<i>C. leptum</i> group	9.86	0.23	10.07	0.30	0.434
sIgA (mg/mL)	67.07	5.75	84.15	6.01	0.009
<b><i>B. longum</i> CECT 7347</b>					
Total bacteria	11.27	0.15	11.08	0.16	0.393
<i>B. fragilis</i> group	9.43	0.20	9.29	0.16	0.583
<i>Enterobacteriaceae</i>	5.90	0.46	6.34	0.43	0.424
<i>Lactobacillus</i> group	8.46	0.21	8.54	0.33	0.858
<i>Bifidobacterium</i> spp.	9.08	0.27	9.16	0.42	0.875
<i>C. coccoides</i> group	9.22	0.23	9.60	0.42	0.469
<i>C. leptum</i> group	10.23	0.23	10.01	0.16	0.447
sIgA (mg/mL)	77.53	4.89	60.83	7.16	0.109

\* Differences between baseline and post-treatment values within the group placebo or *B. longum* CECT 7347 were stabilised at two-sided  $P < 0.05$  using the t-test.

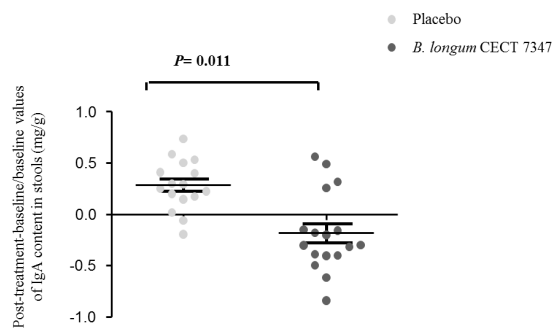
reduced in the placebo group (1.26 [0.08] versus 1.00 [0.04];  $P = 0.006$ ), while *B. longum* CECT 7347 group did not show statistically significant differences (1.19 [0.07] versus 1.16 [0.07];  $P = 0.811$ ). Comparison between groups revealed that gene copy numbers of the *Bacteroides fragilis* group increased significantly in the placebo group compared to the *B. longum* CECT 7347 group ( $P = 0.020$ ) (**Figure 4**). The quantification of sIgA content in faeces

showed that the treatments caused opposite changes within groups, which were statistically significant for the placebo group, showing an increase in faecal sIgA concentration ( $P = 0.009$ ) (**Table 5**). Comparisons between groups revealed that *B. longum* CECT 7347 administration led to significant reductions in faecal sIgA compared to the placebo ( $P = 0.011$ ) (**Figure 5**).

In the *B. longum* CECT 7347 group, decreases in stool sIgA levels correlated



**Figure 4.** Changes in intestinal microbiota composition (log of gene copy numbers/g stools) estimated as the difference between the baseline value and the post-intervention value, divided by the baseline value in placebo (light grey colour) and *B. longum* CECT 7347 (dark grey colour) groups in the three-month intervention parallel to the GFD. Data are expressed as mean and SE. Statistically significant differences between groups were established at two-sided  $P < 0.05$  using the Mann-Whitney  $U$  test.

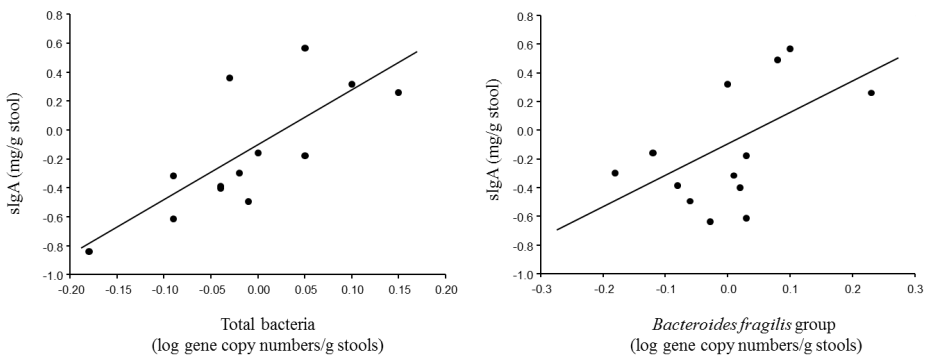


**Figure 5.** Changes in the sIgA content in stools (mg/g) estimated as the difference between the baseline value and the post-intervention value, divided by the baseline value in placebo (light grey colour) and *B. longum* CECT 7347 (dark grey colour) groups in the three-month intervention parallel to the GFD. Bars represent the mean of individual data and SE. Statistically significant differences between groups were established at two-sided  $P < 0.05$  using the t-test.

with decreases in total bacteria and the *Bacteroides fragilis* group gene copy numbers ( $P= 0.005$ ,  $r= 0.729$  and  $P= 0.047$ ,  $r= 0.522$ , respectively, **Figure 6**). No correlations were found between bacteria and other immune parameters either in the placebo group or in the *B. longum* group.

Applying a generalized linear model of analysis, the placebo group showed increases in *Bacteroides fragilis* group numbers of borderline significance compared to the probiotic group ( $P= 0.055$ ). Significant and opposite changes in the sIgA content depending on the treatment were also detected ( $P= 0.031$ ) as well as a time by treatment interaction ( $P= 0.016$ ); faecal sIgA decreased in the *B. longum* CECT 7347 group, while increased in the placebo group. Baseline values for *Bacteroides fragilis* group numbers and faecal sIgA were no different between the two groups ( $P= 0.972$  and  $P=0.416$ , respectively).

lymphocytes (CD3+) and HLA-DR+ T lymphocytes, according to within group comparisons, while the placebo did not. Moreover, there were reduced mature T lymphocytes (CD3+) in between group comparisons with respect to the placebo. Altogether, these results indicate that the bifidobacterial strain exerted a greater effect on these lymphocyte subsets than the GFD alone, which could presumably contribute to better recovery from the inflammatory status associated with the active phase of the disease, which is characterized by increased T cell activation that drives an inflammatory response with increased pro-inflammatory cytokine production (33). Statistically significant correlations between the densities of mucosa CD3+ with the serum tTG 2 antibody levels have been established in untreated and treated CD patients, and the number of intraepithelial CD3+ cells was also



**Figure 6.** Correlation analyses of the changes between baseline and post-treatment values of sIgA concentrations (ng/g stools) and log gene copy numbers of total bacteria and *Bacteroides fragilis* group in the group of children treated with *B. longum* CECT 7347 plus GFD.

associated with the villous height-crypt depth ratio(33). Another study in newly diagnosed CD children (aged 2-5) also reported increased peripheral prevalence of HLA-DR+ cells, which was partially reduced after adherence to a GFD(19). This is in agreement with our findings, pointing to an improved effect of the GFD combined with *B. longum* CECT 7347 intake on this lymphocyte subpopulation. In our study, only regulatory (CD4+Foxp3+) T cells tended to increase in the placebo group, and increased even more in the *B. longum* CECT 7347 group together with helper (CD4+) T cells. Although changes were not statistically significant, this could indicate induction of the regulatory mechanisms controlling the unbalanced inflammatory T cell response associated with CD.

Compared to the placebo, *B. longum* CECT 7347 administration also tends to decrease serum TNF- $\alpha$  concentration, suggesting this bifidobacterial strain could contribute to reducing the inflammatory status of patients to a greater extent than the GFD alone. This finding is also supported by a recent study in an animal model of gliadin-induced enteropathy, reporting that *B. longum* CECT 7347 administration reduced TNF- $\alpha$  production in the small intestinal tissue but increased IL-10 production (34). Statistically significant positive correlations have been reported between IgA-anti-tTG and serum TNF- $\alpha$  in subjects

## DISCUSSION

The influence of administering *B. longum* CECT 7347 orally to children with newly diagnosed CD following a GFD was evaluated in a three-month double-blind randomized placebo-controlled intervention trial. The aim was to assess whether intervention in the gut ecosystem via *B. longum* CECT 7347 administration improved the efficacy of the GFD.

Adherence to the GFD, with and without *B. longum* CECT 7347 supplementation, led to positive effects on growth-related parameters in the children under study, as expected. Furthermore, *B. longum* CECT 7347 administration led to additional improvements, corresponding to greater increases in the height percentile compared to the placebo. Adherence to a GFD is associated with restoring growth, measured as the increase in height percentile and the decrease in weight deficit, as reported in a cohort of 90 children (aged 0.5-7.5) with the disease (31) The effect of GFD on the growth of a cohort of 60 CD children (aged 2.3-10) in a four-year study also showed that the weight-for-height parameter normalized by the end of first year, while height recovery caught up steadily until the 3rd year of adherence to the GFD, revealing faster catch-up of weight than height (32). The administration of *B. longum* CECT 7347 caused significant decreases in mature T

with both type 1 diabetes mellitus and CD (35). Also patients with newly diagnosed CD have been reported to have increased TNF- $\alpha$  serum concentration (36). In the context of CD, TNF- $\alpha$  production, together with IFN- $\gamma$ , are thought to contribute to increasing intestinal epithelial permeability favouring the access of higher antigen loads to the submucosa and, therefore, aggravating CD pathogenesis (37). Recent studies in intestinal cultures *in vitro* and *ex-vivo* have also demonstrated that TNF- $\alpha$  may contribute to exacerbating the pathogenic mechanism of CD by inducing tTG 2 expression, synergically with IFN- $\gamma$  (38).

The present study also demonstrated that in newly diagnosed CD children, imbalances in the microbiota resulting from the GFD were counteracted by parallel administration of *B. longum* CECT 7347. In this respect, in the placebo group the GFD led to significant increases in potentially pathogenic bacteria (*B. fragilis* group and *Enterobacteriaceae*) and reductions in the ratio of harmless to potentially harmful bacteria. In agreement with our present findings, increases in the ratio of *Bacteroides-Prevotella* plus *E. coli/Bifidobacterium* plus *Lactobacillus* group proportions were detected in stools of untreated and treated CD children with a GFD previously (6). In addition, increases in *Bacteroides fragilis* numbers in both stools and duodenal biopsies of patients with active and non-

active disease have been reported previously (7). Furthermore, it was demonstrated that the abundance of the species *Bacteroides fragilis* coding for metalloproteases was increased in both untreated and treated CD patients, which could presumably play a pathogenic role in CD (13). In fact, *Bacteroides fragilis* and the strains producing metalloproteases are frequently involved in opportunistic infections and aggravate colitis in animal models (39). Studies in healthy adults have also shown that the GFD *per se* shifts gut microbiota composition reducing numbers of *Bifidobacterium* spp., *B. longum* and *Lactobacillus* group, and increasing those of *Enterobacteriaceae* and *E. coli* (40). *In vitro* and animal studies have also shown that these microbiota alterations could contribute to pathogenesis in the active phase of the disease as well as to the lack of complete restoration of immune homeostasis in GFD-treated patients (10, 12, 14). However in the present study statistically significant changes specifically in numbers of *Bifidobacterim* spp. were not detected neither associated with the adherence to the GFD nor with the probiotic administration. It has been reported that changes in microbiota resulting from a dietary intervention depend among other factors on the initial microbiota composition and, accordingly, previous studies have indicated that changes in bifidobacterial numbers can be minor after probiotic or prebiotic

administration in subjects with high initial bifidobacterial counts (41) as it was the case for our study. Also, according to our previous studies, the GFD does not favour the growth of bifidobacteria (40).

The changes induced by *B. longum* CECT 7347 administration in the intestinal microbiota were parallel to decreases in faecal sIgA concentration. The sIgA is considered to be the most likely host secretion to affect the localization, growth and composition of gut microbiota, and can restrict colonization of harmful bacteria (42, 43). Nevertheless, it is also speculated that gut microbiota composition may influence sIgA concentration and specificities as a result of a bi-directional cross-talk between the host and its microbiota (42, 43). The fact that *B. longum* CECT 7347 administration caused a sIgA reduction that was statistically correlated with the lower gene copy numbers of total bacteria and the *Bacteroides fragilis* group would suggest a reduction in the host sIgA-mediated protective response against luminal bacterial antigens due to restoration of the gut ecosystem with decreases in harmful bacteria (44). A recent randomized, double-blind placebo-controlled study evaluated the effect of *B. infantis* NLS in untreated CD patients consuming gluten to establish the effect of the probiotic independently of the GFD (45). The beneficial properties of *B. infantis* NLS included the reduction of gastrointestinal symptoms,

specifically indigestion, constipation and reflux with borderline significance. However, it did not improve diarrhoea or abdominal pain, nor modify intestinal permeability or the pro-inflammatory status as reflected by the analysis of serum cytokines and chemokines (45). Despite the experimental differences, it is presumable that the mechanism behind the effects exerted by *B. infantis* NLS differ from those exerted by *B. longum* CECT 7347, as the latter influences inflammatory markers and the gut microbiota and host-related defence mechanisms.

The study reported herein has some limitations partly inherent to exploratory interventions. These include: small population size, lack of previous data for power calculation, inclusion of children with a wide age range, which could increase the variability of immune parameters determined, and a relatively short duration to detect clinical effects. Nonetheless, the findings are interesting enough to encourage further well-powered intervention studies, which would provide sound data on the efficacy of this bifidobacterial strain and shed light on its mode of action in ameliorating inflammatory conditions such as CD.

In conclusion, the oral administration of *B. longum* CECT 7347 together with the GFD led to shifts in the intestinal microbiota, characterized by reductions in potentially pro-inflammatory bacteria (*B. fragilis*

group) related to CD in previous human observational studies as well as faecal sIgA. Likewise, *B. longum* CECT 7347 administration led to reductions in activated T lymphocytes and also tended to reduce inflammatory markers (TNF- $\alpha$ ) that may contribute to recovery of the immune homeostasis in patients.

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## SUPPLEMENTARY TABLES

**Sup. table 1:** Sequence of the 16S rRNA gene target primers used for real time quantitative PCR analyses.

Targeted bacteria	Sequence (5'-3')	T <sup>a</sup> anne.	Amplicon size (pb)	Reference
Total bacteria	ACT CCT ACG GGA GGC AGC AG CTA TTA CCG CGG CTG CTG GCAC	59	200	Matsuki <i>et al.</i> , 2002
<i>Bacteroides fragilis</i> group	ATA CGG AGG ATC CGA GCG TTA CTG TTT GAT ACC CAC ACT	48	287	Matsuki <i>et al.</i> , 2002
Enterobacteriaceae	CAT TGA CGT TAC CCG CAG AAG AAG C CTC TAC GAG ACT CAA GCT TGC	63	195	Bartosch <i>et al.</i> , 2004
<i>Lactobacillus</i> group	AGC AGT AGG GAA TCT TCC A ATT YCA CCG CTA CAC ATG	61	340	Walter <i>et al.</i> , 2000
<i>Bifidobacterium</i> spp.	CTC CTG GAA ACG GGT GG GGT GTT CTT CCC GAT ATC TAC A	55	550	Matsuki <i>et al.</i> , 2002
<i>C. coccoides</i> group	AAA TGA CGG TAC CTG ACT AA CTT TGA GTT TCA TTC TTG CGA A	50	438	Matsuki <i>et al.</i> , 2002
<i>C. leptum</i> group	GCA CAA GCA GTG GAG T CTT CCT CCG TTT TGT CAA	50	239	Matsuki <i>et al.</i> , 2004

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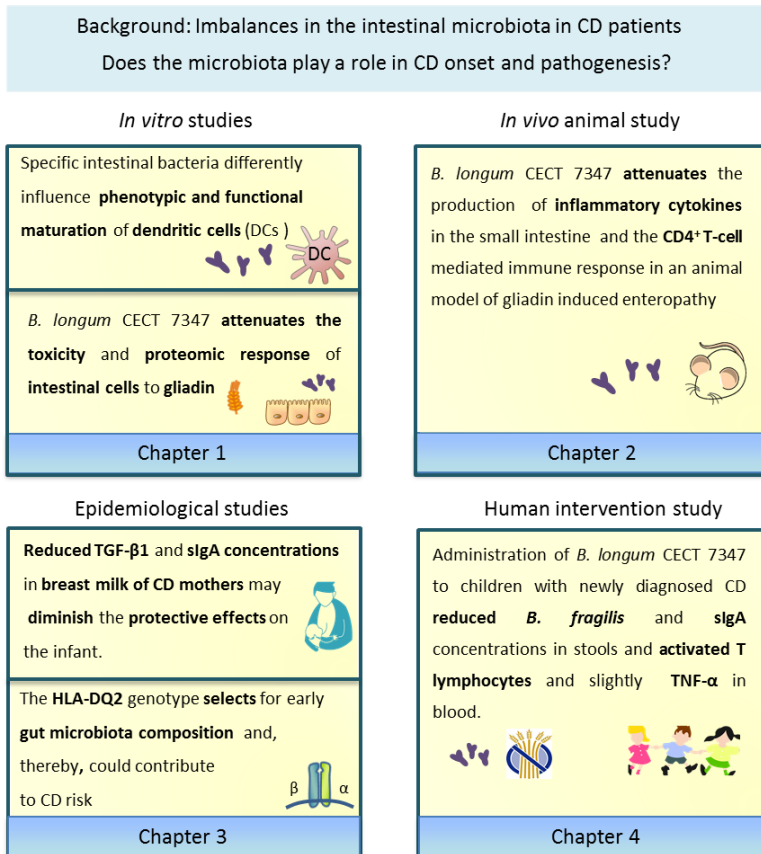
**Sup. table 2.** Nutrient intake in the placebo and *B. longum* CECT 7347 groups at baseline and during the three-month intervention.

	Baseline		Treatment period		* <i>P</i> value
	Mean	SE	Mean	SE	
<i>Placebo</i>					
Energy (Kcal)	1904.98	113.40	2144.60	326.41	0.476
Protein (g)	67.50	8.00	85.92	17.01	0.589
Lipids (g)	92.34	5.82	104.84	17.28	0.673
Carbohydrates (g)	200.98	17.15	214.34	27.58	0.576
Phosphorous (mg)	1053.74	141.81	1457.70	400.54	0.458
Magnesium (mg)	181.04	11.69	190.58	14.81	0.583
Calcium (mg)	939.42	174.55	1057.02	258.03	0.767
Iron (mg)	8.46	0.71	10.32	0.96	0.103
Zinc (mg)	6.66	0.75	7.68	1.04	0.553
Vitamin C (mg)	81.86	16.94	77.68	13.30	0.848
Vitamin A (retinol) (µg)	718.04	142.76	2129.40	712.16	0.119
Vitamin D (µg)	2.10	0.83	3.98	1.73	0.443
Vitamin E (mg)	7.30	1.17	9.98	1.89	0.171
Fibre (g)	10.80	1.35	8.56	2.45	0.355
<i>B. longum</i> CECT 7347					
Energy (Kcal)	2079.57	523.42	2087.82	414.30	0.989
Protein (g)	78.67	17.10	78.75	15.02	0.272
Lipids (g)	107.17	30.65	95.82	22.39	0.447
Carbohydrates (g)	200.10	45.74	227.62	46.41	0.966
Phosphorous (mg)	1130.05	204.61	1139.77	208.90	0.917
Magnesium (mg)	171.42	22.67	197.78	48.43	0.495
Calcium (mg)	795.60	110.08	825.55	138.82	0.493
Iron (mg)	11.37	1.91	13.23	3.40	0.526
Zinc (mg)	9.08	1.98	9.62	2.25	0.672
Vitamin C (mg)	78.55	11.78	96.03	34.09	0.519
Vitamin A (retinol) (µg)	1298.32	315.24	1237.83	395.49	0.915
Vitamin D (µg)	3.80	1.24	5.42	1.37	0.319
Vitamin E (mg)	9.12	1.33	9.33	1.80	0.903
Fibre (g)	12.32	3.12	9.4	1.89	0.161

\*Differences between baseline and post-treatment within the groups (placebo or *B. longum* CECT 7347) were established at  $P < 0.05$  using the t-test.

## IV – GENERAL DISCUSSION

The findings of this Doctoral Thesis, which are presented in the previous four chapters, respond to the common objective of progressing in the understanding of the role of the intestinal microbiota and specific bacterial components in the pathological process of CD, and contributing to the disease prevention and treatment by identifying factors that beneficially modulate gut microbiota composition and function. **Figure 7** shows a schematic representation of the different studies carried out during this Thesis and their main findings and scientific contribution.



**Figure 7:** Schematic representation of the studies included in the present Thesis

### **Genetic and environmental factors together modulate the early intestinal colonization and may influence the CD onset**

The expression of the HLA-DQ2 or HLA-DQ8 heterodimers and the gluten intake do not explain why some subjects develop CD meanwhile others not (27, 28). Among the environmental factors possibly involved in CD in addition to gluten, breast feeding is the most intensively investigated but without conclusive results. A protective role of breast-feeding has been reported, mostly based on a few observational, retrospective studies (92-95) which were summarized in a meta-analysis (55) and a systematic review (96). However, not all the studies support a protective effect (97) and any of them clarifies whether breast-feeding provides permanent protection against CD or simply delays the onset of CD development.

In the first study included in **Chapter 3**, the differences between breast-milk composition of healthy mothers and mothers with CD on a GFD have been analyzed for the first time. Mothers with CD presented a decrease in several immune markers (IL-12p70, TGF- $\beta$ 1 and sIgA) and in *Bifidobacterium* spp. in their breast milk. These differences might influence the protective effects of breast-feeding on the infant since it constitutes a source of immunoglobulins and cytokines that compensates the immature immune system of the neonate. These differences in the breast milk composition could also explain, at least in part, the controversy across studies regarding the effects of human milk in the protection against CD. Mothers with the disease also presented a reduction in the *Bifidobacterium* spp. 16S rRNA gene copy numbers, which could mean a reduction in the transfer of protective bacteria to the newborn. Although more studies are needed, it could be speculated that a reduction in immune protective components (sIgA and TGF- $\beta$ 1), involved in oral tolerance development, as well as of potential beneficial bacteria could influence the development of the immune system of the neonate and the intestinal colonization pattern, that may influence the development of CD later in life.

In the second study of **Chapter 3**, we have also analyzed the influence of the HLA-DQ genotype on the intestinal microbiota composition of healthy infants

at family risk of developing CD. This study is part of a larger prospective cohort study aiming to unravel whether alterations in the intestinal microbiota could precede the onset of CD. Our first studies with this cohort of infants using real time qPCR showed that the milk-feeding practices and the HLA-DQ2/DQ8 genotype influence the intestinal colonization (69). To progress beyond this first observation, in the study included in the present Thesis we have analyzed the influence of the genotype on the microbiota in a sub-group of 22 infants including only exclusively breast-fed and vaginally delivered infants to reduce the number of confounding variables, and using 16S rRNA gene pyrosequencing. We have observed that the infants with a high genetic risk (HLA-DQ2) of developing CD showed increased proportions of Firmicutes (*Clostridium sensu stricto* and unclassified Clostridiaceae and *Gemella*) and Proteobacteria (*Raoultella* and unclassified Enterobacteriaceae) and reduced proportions of Actinobacteria (*Bifidobacterium*) compared to infants with a low genetic risk (non-HLA-DQ2/DQ8). This observation let us to confirm that the HLA-DQ2 genotype *per se* influences the intestinal microbiota composition, even though the mechanism underlying the association of these imbalances with the later development of the CD remains unknown.

HLA genotype encodes for proteins expressed on the surface of cells that bind to antigen fragments for their presentation to the T cells. Thus, when CD patients ingest gluten, HLA molecules recognize gluten peptides and activate T CD4<sup>+</sup> cells, triggering an aberrant immune response. In order to propose a mechanism by which HLA selects for intestinal microbiota, we can speculate that HLA molecules could present phagocytized antigens of intestinal bacteria, which may then be presented to T cells. Depending on the antigen presented, effector T-cell activation could contribute to regulating the gut microbes colonizing the gut by activating B cells to secrete protective antibodies directly into the gut mucosa and lumen (98). Bacterial antigens presented via MHC II molecules, could also lead to T cell maturation into effector cells (Th1, Th2 or Th17) or Foxp3<sup>+</sup>Treg cells with immunosuppressive activity, which could contribute to developing tolerance towards components of the intestinal microbiota. In this context, studies in rodents indicate that the repertoire of thymus-derived Treg cells, which constitute

most Treg cells in all lymphoid and intestinal organs including the colon, is heavily influenced by microbiota composition, thus supporting this hypothesis (99).

In summary, from the results presented in **Chapter 3**, we can conclude that breast-milk from CD mothers and healthy mothers differs in composition and that this could reduce the protective effects of breast-feeding and the transfer of bifidobacteria to the offspring increasing CD risk. In addition, the HLA-DQ2 genotype influences the intestinal microbiota composition of infants at family risk of developing CD, especially reducing the proportion of Actinobacteria (*Bifidobacterium*) in the gut, which could also be a factor that confers less resilience to disease in this population group. These observations, together with the evidence that bifidobacteria numbers were reduced in CD patients reported previously (reviewed in 31), seem to indicate that this bacterial genus might play a protective role in CD.

### ***B. longum* CECT 7347 as potential probiotic for CD**

The associations between CD and intestinal dysbiosis and the role attributed to some probiotics in the regulation of the gut barrier function and immune response have motivated investigations of the potential beneficial effect of dietary intervention based on these functional ingredients (reviewed in 100). Some of the studies included in this Doctoral Thesis have been focused on the evaluation of the protective role of *B. longum* CECT 7347 and its mechanisms of action in CD, which are reported in **Chapters 1, 2** and **4**.

*B. longum* CECT 7347 (also called IATA-ES1) was isolated in 2007 from a three-month old healthy breast-fed infant. This strain of the specie *Bifidobacterium longum* was selected for its ability to withstand the passage through the digestive tract, a very hostile environment where the acidity and secretion of bile and other compounds kills many bacteria, and also for its *in vitro* ability to inhibit pathogens isolated from CD patients, immunoregulatory properties and ability to hydrolyse gluten peptides (e.g. the 33-mer). Later studies with this bacterium also demonstrated that *B. longum* CECT 7347 could counteract the pro-inflammatory effects induced by the CD patients' microbiota in PBMC cultures. In



particular the co-incubation of *B. longum* CECT 7347 with PBMCs reduced the production of pro-inflammatory cytokines (IFN- $\gamma$  and TNF- $\alpha$ ) induced by the altered microbiota of CD patients and increase the release of the anti-inflammatory cytokine IL-10 counteracting the Th1-type response (87). In a later study, *B. longum* CECT 7347 was also demonstrated to be able to hydrolyze gliadin peptides reducing their toxicity and inflammatory properties in Caco-2 cells (91).

The present Thesis has progress in the understanding of the mechanisms by which *B. longum* CECT 7347 could protect against CD using different and complementary experimental approaches. The **Chapter 1** reported the *in vitro* studies, the **Chapter 2** described the *in vivo* pre-clinical studies in an animal model of the enteropathy and the **Chapter 4** included a double-blind randomized intervention study in children newly diagnosed with CD to demonstrate the clinical efficacy of *B. longum* CECT 7347.

The articles of **Chapter 1** were conceived to study whether some specific components of the intestinal microbiota (especially some *Bifidobacterium* strains) could contribute to ameliorating the response to gluten peptides using *in vitro* models. To do so, monocytes derived dendritic cells (MDDCs) and Caco-2 cultures were chosen as both cells types are located at the interface between the host and the microbiota, and regulate intestinal barrier function and innate immune responses.

In the first study of **Chapter 1**, we concluded that specific components of the gut microbiota could define the role of dendritic cells (MDDCs) in T cell activation and the progression of CD, via their influence in the maturation of MDDCs and their interactions with epithelial cells (Caco-2). Specifically, some enterobacteria exerted pathogenic effects similar to the ones exerted by gliadin, contributing to the MDDCs maturation and the induction of pro-inflammatory cytokine release. In contrast, the two *Bifidobacterium* strains tested (*B. bifidum* CECT 7365 and *B. longum* CECT 7347) did not cause gliadin-induced morphological changes of MDDCs and showed reduced inflammatory cytokine release. Furthermore, *B. longum* CECT 7347 reduced the IFN- $\gamma$  release induced by gliadins and increased IL-10 secretion when both stimuli were combined. Also this bacterium reversed the gliadin-reduced ZO-1 expression in Caco-2 cells, which alters permeability.

Altogether, these data showed that *B. longum* CECT 7347 could play a role in CD through the polarization of the immunological response and strengthening the gut barrier function, making this strain the most interesting one for further studies.

In the second *in vitro* study included in **Chapter 1**, we evaluated the potential protective properties of *B. longum* CECT 7347 by contributing to the digestion of gliadin peptides and reducing their toxicity on Caco-2 cells using a proteomic approach. To do so, the proteome of Caco-2 cells exposed to gliadin peptides digested *in vitro* in the presence or absence of *B. longum* was evaluated. We observed a higher number of proteins with altered expression when Caco-2 cells were exposed to gliadin digested without *B. longum*, indicating a higher damage, than when gliadin was digested in the presence of the *Bifidobacterium*. Some of these proteins were involved in the maintenance of the cell cytoskeleton, cell apoptosis and interactions with the immune cells. Besides, this differential proteomic response was associated with a reduction in cell viability and an increase in TNF- $\alpha$  release. By contrast, when Caco-2 cells were exposed to gliadin digested in the presence of *B. longum* CECT 7347, a lower number of proteins were altered and no differences in the inflammatory markers were detected compared to the control. Thus, it was concluded that *B. longum* CECT 7347 reduced the toxic and the inflammatory effects of gliadin-derived peptides generated during a simulated gastrointestinal digestion. In addition to the proteolytic effect on gliadin peptides, a role of other molecules released by *B. longum* during the digestion process cannot be discarded.

In **Chapter 2**, we have evaluated the potential effects of *B. longum* CECT 7347 in an *in vivo* model of gluten induced enteropathy in newborn rats fed gliadin and sensitized with IFN- $\gamma$ . We selected this animal model because it was described that reproduces the CD4<sup>+</sup> T cell mediated enteropathy defined as hyperplastic-infiltrative similar to the one described in CD patients and the CD symptoms (101). The results confirmed that the sensitization with IFN- $\gamma$  was necessary to partially reproduce the disease. In previous studies was also shown that sensitization of animals with IFN- $\gamma$  was necessary to cause mucosa damage

and immunological changes that resemble the enteropathy observed in human, as IFN- $\gamma$  increases the paracellular transport and the activation and priming of macrophages (102, 103). In sensitized newborn rats fed gliadin, the administration of *B. longum* CECT 7347 increased the NF- $\kappa$ B expression and IL-10 concentration and reduced TNF- $\alpha$  production in the small intestine. The administration of *B. longum* CECT 7347 also reduced CD4<sup>+</sup> and CD4<sup>+</sup>/Foxp3<sup>+</sup> cell populations and increased CD8<sup>+</sup> T cell populations in peripheral blood from sensitized gliadin-fed animals. These results suggest that the *Bifidobacterium* partially counteract the inflammatory response locally in the gut. However, results from animal models have limitations and the potential beneficial effects should be interpreted with caution.

Finally, **Chapter 4** includes the most conclusive study as it evaluated whether *B. longum* CECT 7347 could help improve the recovery of children with newly diagnosed CD when administered in addition to the GFD.

When compared between groups after the 3 months of intervention, the administration of *B. longum* CECT 7347 caused a decrease in peripheral activated CD3<sup>+</sup> T lymphocytes and a slightly reduction in the TNF- $\alpha$  concentration. In the gut, the administration of *B. longum* was associated with a reduction of sIgA and *Bacteroides fragilis* numbers, which could contribute to restoring the gut ecosystem since bacteroides are known to be increased in the microbiota of CD patients (64). The production of sIgA plays a protector role as it binds harmful antigens (allergens or pathogens) and prevents the colonization and invasion of pathogens, as it can be the case for pathobionts of the genus *Bacteroides* (104). However, in active CD patients the sIgA may not play a protective role as the abnormal retro-transport of the complex IgA-gluten peptide by the CD71 receptor has let to consider sIgA as a “Trojan horse” able to promote the entrance of gluten peptides (104, 105). Thus, it could be speculated that the reduction observed in the children who received *B. longum* CECT 7347 may reduce the translocation of gluten to the lamina propria, when eventually ingested. It can also be a consequence of reductions in the total load of pathobionts in the intestine, such as *Bacteroides fragilis* group.

Despite no differences in the incidence of gastrointestinal symptoms were reported, our findings suggest that *B. longum* CECT 7347 could help to restore the intestinal ecosystem of CD patients. To date, two more randomized, double-blind placebo-controlled human intervention trials have been conducted in CD patients (106, 107). One of this trials has evaluated the effects of the administration of *B. infantis* NLS to untreated CD patients independently of the GFD. This study reported an improvement in some gastrointestinal symptoms (indigestion and constipation) after the administration of *B. infantis* NLS, but no differences in the permeability or the pro-inflammatory cytokines were found (106). The more recent intervention study published evaluated the effect of a strain combination (*B. breve* BR03 and *B. breve* B632), as compared to a placebo in children with CD on a GFD. In this case, the two *B. breve* strains decreased the production of the pro-inflammatory cytokine TNF- $\alpha$  in children with CD on a GFD (107) as detected in our study.

Although these three studies report promising results since the interventions with specific probiotics strains ameliorate the symptoms or the inflammatory status of CD patients, the reproducibility of the effects in larger population groups should be confirm to be able to draw firm conclusions and make recommendations about their use in practice.

## V – CONCLUSIONS

1. CD mothers' breast milk is characterized by a reduced abundance of immunoprotective compounds (TGF- $\beta$ 1 and sIgA) and DNA sequences from *Bifidobacterium* spp. The reduction in these components could theoretically diminish the protective effects of breast-feeding on the child's future risk of developing CD and explain the controversy across previous epidemiological studies.

2. Infants with high genetic risk of developing CD (HLA-DQ2) have higher proportions of Firmicutes (*Clostridium* species) and Proteobacteria (Enterobacteriaceae) and lower proportions of Actinobacteria (*Bifidobacterium* species) than those with low genetic risk (non-HLA-DQ2/DQ8-carriers) in exclusively breast-fed infants. Therefore, the HLA-DQ2 genotype *per se* influences the early gut microbiota composition in infants at family risk of developing CD and could be an additional factor that contributes to CD onset.

3. Specific components of the gut microbiota may influence phenotypic and functional maturation of DCs differently, and their interactions with epithelial cells aggravating (enterobacteria) or protecting (bifidobacteria) from the adverse effects of gluten peptides *in vitro*.

4. *B. bifidum* CECT 7365 and *B. longum* CECT 7347 did not cause gliadin-induced morphological changes, and induced adhesion and spreading ability of MDDCs and inflammatory cytokine production to a lesser extent than the enterobacteria. *B. longum* CECT 7347 reduced IFN- $\gamma$  production induced by gliadins and increased IL-10 secretion and also reversed the gliadin-reduced ZO-1 expression in Caco-2 cells, suggesting that protects from the adverse effects of gluten

5. The presence of *B. longum* CECT 7347 during the *in vitro* digestion of gliadin reduces its toxicity, measured as increased cell viability and reduced pro-inflammatory cytokine secretion. It also modifies the proteomic response of intestinal epithelial cells (Caco-2 cells) to gliadin, reducing the altered protein expression.

6. In an animal model of gliadin-induced enteropathy, the administration of *B. longum* CECT 7347 attenuated the production of inflammatory cytokines in the small intestine and the increased CD4<sup>+</sup> T-cell mediated immune response in the periphery.

7. In children newly diagnosed with CD, the oral administration of *B. longum* CECT 7347 together with the GFD reduced the numbers of potentially pro-inflammatory bacteria (*B. fragilis* group) related to CD in previous human observational studies and faecal sIgA concentration. It also led to reductions in activated T lymphocytes and slightly in inflammatory markers (TNF- $\alpha$ ) that may contribute to recovery of the immune homeostasis in CD patients.

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# Abbreviations

ACN	Acetonitrile
Akt/mTOR	Protein kinase B/mammalian target of rapamycin
APAF-1	Apoptotic protease activating factor 1
APC	Antigen presenting cells
APTS	9-aminopyrene-1,4,6-trisulfonate)
B	To refer proteins modified by <i>B. longum</i> CECT 7347
CD	Celiac disease
CE	Common Era
CECT	Coleccion española de cultivos tipo
CE-LIF	Electrophoresis-laser-induced fluorescence
CFU	Colony forming units
CHCA	$\alpha$ -Cyano-4-hydroxycinnamic acid
CI	Cell index
CK	Cytoskeletal
CSIC	Consejo Superior de Investigaciones Científicas
Ct	Cycle threshold
2-D	Two dimensionals
DC	Dendritic Cell
DFL	Difucosyllactose
DF LNH	Difucosyllacto-N-hexaose
DGGE	Denaturing gradient gel electrophoresis
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothitol
EDTA	Ethylenediaminetetraacetic acid
EMA	Endomysial antibodies
ESPGHAN	European Society for Pediatric Gastroenterology, Hepatology and Nutrition
FISH	Fluorescence <i>in situ</i> hybridization
FITC	Fluorescein isothiocyanate
FL	Fucosyllactose
F LNH	Fucosyllacto-N-hexaose
Fuc	Fucose
FUT	Fucosyltransferase
G	To refer proteins modified by gliadin without <i>B. longum</i>
Gal	Galactose
GB	To refer proteins modified by gliadin with <i>B. longum</i>
GFD	Gluten-free diet
Glu	Glucose
GTP	Guanosine triphosphate
HLA	Human Leukocyte antigen
HMOs	human milk oligosaccharides
HR	High risk
IAA	Iodoacetamide
IELs	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPG	Immobilized pH gradient

Le	Lewis
LGALS3	Lectin galactoside-binding soluble 3
LIF	Laser-induced fluorescence
LNDFH	Lacto-N-difucosylhexaose
LNFP	Lacto-N-fucopentose
LNT	Lacto-N-tetraose
LR	low risk
MDDCs	Monocytes Derived Dendritic Cells
MRSC	Man-Rogosa-Sharpe cysteine
MS	Mass Spectrometry
N-Acetyl	N Acetylglucosamine
NCBI	National Center for Biotechnology Information;
Neu5Ac	sialic acid
NF- $\kappa$ B	Nuclear factor kappa-B
NGS	Next generation sequencing
NR	Neutral red
OTUs	Operational taxonomic units
P50	percentile 50
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	phycoerythrin
<i>pI</i>	isoelectric point
PSLG1	P-selectin glycoprotein ligand 1
qPCR	quantitative PCR
RDP	Ribosomal Database Project ();
RT-qPCR	Real time Reverse transcription chain reaction
SDS	Sodium dodecyl sulfate
Se	Secretor
sIgA	Secretory immunoglobulin A
SL	Sialyllactose
SPTA	Spectrin alpha chain
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TFA	trifluoroacetic acid
TGF	transforming growth factor
Th	T helper
TJ	Tight Junction
TNF	Tumor necrosis factor
TLRs	Toll-like receptors
tTG	Tissue transglutaminase
tTG-2	tissue transglutaminase 2
TTGE	temperature gradient gel electrophoresis

## List of publications

This Doctoral Thesis comprises six original research papers in indexed journals. Besides, some reviews and book chapters related to the field of this Thesis have been published during the pre-doctoral period and are listed below.

De Palma G, Kamanova J, Cinova J, Olivares M, Drasarova H, Tuckova L, Sanz Y (2012). Modulation of phenotypic and functional maturation of dendritic cells by intestinal bacteria and gliadin: relevance for celiac disease. *J Leukoc Biol* 92, 1043-1054.

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### BOOK CHAPTERS

Olivares M, Sanz Y. Intestinal microbiota and Celiac Disease. In “Advances in the understanding of gluten related pathology and the evolution of gluten-free foods”. Ed: Rodrigo L, Peña S, Arranz E, Fernández-Bañares F, Rosell CM. Barcelona, Spain. OmniaScience, 2015, in press.

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