Chapter 25

Scientific Design of a Dairy Product aimed at Celiac Patients

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Abstract

After receiving a celiac disease diagnosis, patients need to follow a gluten-free diet. The technological bases of gluten-free products are focused on generating gliadin-free products, without providing any other nutritional benefits. Quite recently we have developed a milk supplement called *Proceliac* which aims to change this trend in the design of products for celiac patients. The basis of this product is a probiotic called ES1 that has shown strong anti-inflammatory effects in both experiments with human cell cultures and in preclinical animal experiments. The food safety of the ES1 probiotic has been evaluated following the World Health Organization guidelines. Moreover, its genome has been fully sequenced to ensure the absence of genes encoding conflicting proteins. Finally, two clinical trials on healthy adults and children with celiac disease at the beginning of gluten-free diet have been performed with excellent results that indicate this strain's ability to equilibrate the gut microbiota of celiac patients.

1. Introduction

1.1. Celiac Disease

Celiac disease (CD) is an autoimmune disease that occurs when genetically predisposed individuals ingest α-gliadin peptides from wheat or other cereals.[1,2] Clinical manifestations include intestinal inflammation symptoms and nutrient malabsorption, along with severe mucosal damage.[3] This inflammation occurs because, after consumption, α-gliadin is partially degraded by digestive proteases yielding proteolysis-resistant oligopeptides due to its high glutamine and proline content.[4,5] These peptides trigger the inflammatory immune response leading to the disease symptoms.[6-11] Along with these inflammatory effects, it should be noted that individuals with celiac disease suffer significant changes in intestinal microbiota, since it has a greater quantity of strains belonging to the *Bacteroides* and *Clostridium* genera, and a lower bifidobacteria proportion.[12-17]

CD incidence is estimated at 1% of the population, although it is estimated that, for every case diagnosed, there may be between 7 and 11 undiagnosed cases.[18] There is no therapeutic treatment for celiac disease. Therefore, the celiac individual must follow a lifelong gluten-free diet.[19] The global market for gluten-free food is widespread and grows beyond what was thought in market research early this decade. It is estimated that in 2012 in the U.S.A. alone sales of more than 4,200 million dollars were generated, and this figure is expected to rise to more than 6,500 million by the end of 2017. The 2012 annual compound growth rate stood at 28% and, more importantly, the number of consumers of such products increased from 15% to 18% in just two years.[20] Still, it must be remembered that the basis of this dietary offer is "no gluten" and that in no case products have been offered which, while lacking it, have additional nutritional or functional characteristics of interest for the celiac patients. So, a few years ago, the Asturian Milk Central, the Scientific Research Board of Governors (CSIC) and Biopolis SL, a biotechnological company, became interested in this problem and decided to tackle a research and development project that would yield a new product that would benefit the celiac patient's nutrition. It was a long-term wager, full of unknowns, but worthwhile (Figure 1). This product would be gluten-free and also have a nutritional profile that could help maintain the health of a celiac patient. The following pages describe the development of said product, called *Proceliac*.

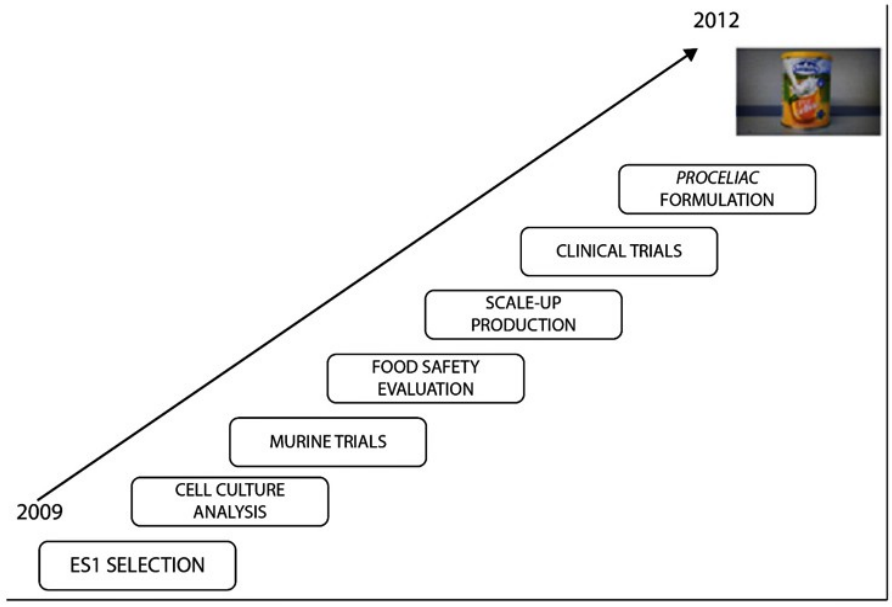

Figure 1. Proceliac development stages.

2. Selection of the ES1 Probiotic

Proceliac is based on a bacterium belonging to the genus *Bifidobacterium longum*. This bifidobacteria was isolated at the Institute of Agrochemistry and Food Technology, CSIC (IATA-CSIC) by Dr. Yolanda Sanz's group.[21] After screening hundreds of bifidobacteria isolates from the feces of children under three months of age, healthy and breastfed, they found a strain which they named ES1, which had the general properties of a probiotic. On one hand, this strain resisted extreme acidity values and high bile salt concentrations, on the other hand, it survived the passage through the digestive tract, as found in human volunteers that ingested it. It was also able to partially adhere to the surface of human intestinal cells. Besides, it also partially inhibited the growth of several bacterial pathogens found in excess in the intestinal microbiota of celiac patients.

All these properties were important since they conferred a probiotic character to the ES1 strain. Even more important was the fact that this strain partially degraded many of the gliadin peptides responsible for triggering celiac inflammation, as demonstrated in an experiment with suspension cultures of human intestinal epithelial Caco-2 cells to which gliadin samples were added which had been previously subjected to *in vitro* gastrointestinal digestion. The whole was co-incubated with the ES1 strain or other bifidobacteria and the resulting peptide mixtures for each case subjected to an RP-HPLC-ESI-MS/MS analysis. This helped determine the generated degraded peptides which were evaluated according to their toxicity. The results indicated that each bifidobacteria strain produced a distinct set of degraded gliadin peptides and that ES1 gliadin was the only one that did not produce α-β-gld (122-141) or α-β-gld (158-164), which cause inflammation by interacting with the CXCR3 receptor. Consequently, no cytotoxicity was detected only in the sample containing this probiotic strain.[22]

3. ES1 Strain Anti-inflammatory Capacity

In the IATA-CSIC laboratories it has been have shown that this strain is able to induce an anti-inflammatory response in three different cell models. The first study was conducted in collaboration with the University Hospitals La Fé and General and from the University of Valencia. Feces of celiac children, with symptoms and without symptoms, and of healthy children, were taken which were incubated with the ES1 probiotic or with a placebo. In turn, the whole was co-incubated with peripheral blood mononuclear cells from healthy adults. The results indicated that the feces of celiac children exposed to a placebo yielded a significant increase in the synthesis of the TNF-α inflammation-stimulating cytokine. In increase in CD86 production and decreased IL 10 anti-inflammatory interleukin synthesis and CD4 expression were also detected. In the case of feces from children with symptoms, a high IFN-γ expression indicative of an inflammation peak was detected. By contrast, feces incubated with the ES1 probiotic had no increased synthesis of all these inflammatory markers and, conversely, more IL-10 anti-inflammatory cytokine was synthesized.[23]

Subsequently, the IATA-CSIC group used another cellular model, Caco-2 human intestinal epithelial cells, which were treated with gliadin hydrolyzate in the presence or absence of the probiotic strain. Transcriptomics were used to quantify the expression of several encoding genes related to inflammatory response such as the CXCR3 receptor, NF-κβ and TNF-α, and the production of proinflammatory markers such as IL-1β, p50 was analyzed, as well as NF-κβ and TNF-α themselves. The results were very similar to those of the previous study, since epithelial cells co-incubated with the ES1 probiotic showed a decrease in the transcription of inflammation marker genes and, consequently, a decrease in the detection of the same.[22]

Finally, the effect of the addition of ES1 strain to human dendritic cell cocultures, Caco-2 human intestinal epithelial cells and gliadin hydrolyzate was studied. In this case, two enterobacteria isolated from the feces of celiac patients (CBL2 *Escherichia* coli and CBD8 *Shigella*) were used as controls. This work was performed within the framework of a collaboration between the IATA-CSIC and the Department of Immunology of the Czech Republic Academy of Sciences. The pathogenic microorganisms induced cytological changes in dendritic cells, such as podosoma dissolution, activation of adhesion and spreading, and also a peak in several inflammatory markers such as IFN-γ, IL-12 and TNF-α. These changes were not detected when adding the ES1 probiotic strain which also did not activate adhesion, reduced the CD40, CD86 and IFN-γ expression and increased anti- IL-10 inflammatory cytokine secretion.[24]

Finally, a proteomic study was performed in order to analyze the Caco-2 human intestinal epithelial cell secretome cultured with gliadin hydrolyzate in the presence of the ES1 probiotic or a placebo. Using 2DE and MALDI-TOF, a greater number of secreted proteins were detected in the case of the placebo than for ES1 strain. Most of these proteins were associated with cytoskeleton disorganization, inflammation and apoptosis. In the case of the group treated with the ES1 probiotic these proteins were not detected. On the contrary, the presence of proteins related to cell survival and function and calcium homeostasis was found. All these results were indicative of a gliadin toxicity decrease.[25]

4. Assays in Experimental Animals

Despite multiple efforts, unfortunately there is no animal model for celiac disease.[26] Following these tests in cell models, IATA-CSIC researchers decided to move to a study in newborn rats in which intestinal enteropathy was induced by treatment with IFN-γ. These rats were fed with gliadin and placebo or with the ES1 probiotic. After the test, the animals were sacrificed and a histological jejunum examination was performed, analyzing the expression of the gene encoding NFκβ and the production of various cytokines. The production of leukocyte populations and T-cells were also studied. Analysis of the results indicated that the group of rats that received the placebo showed changes in the structure of the intestinal epithelium, mainly a greater cellular infiltration, reduced villi width and enterocyte height reduction. By contrast, the group of rats that had ingested the ES1 probiotic had improved epithelial architecture. In addition, the rats that ate placebo had increased T CD4+, CD4+/Foxp3+ and CD8+ cells. In addition, it was found that ES1 probiotic intake reduced TNF production and increased the anti-inflammatory cytokines such as IL-10.[27] In a later study using the same animal model, an analysis was performed on the proteome of jejunal sections from animals sensitized with IFN-γ or not, which had been fed gliadin in the presence or absence of the ES1 probiotic, there being only significant differences in non-sensitized animals that had ingested ES1 compared with those who had not done so.[28]

5. Probiotic Strain ES1 Food Safety

All these encouraging results, determined the beginning of the ES1 strain food safety study which was undertaken at Biopolis SL, following the by FAO and the World Health Organization recommendations.[29] In the first stage the ES1 strain's production of toxic compounds such as biogenic amines or deconjugated bile salts was evaluated. The D and L-lactic acid productivity and their relationship where also quantified as well as the resistance levels to many antibiotics intended for hospital use. No problematic values were detected.

During the second phase, and in collaboration with the Pasteur Institute of Montevideo, an acute toxicity study was made using normal and immunosuppressed mice by means of pharmacological treatment. Both groups were fed a placebo or the ES1 probiotic. After a 9-day intake, no physiological problems were detected. After this time the animals were sacrificed and a pathological examination of all organs was performed; no abnormalities were detected. Finally, an analysis was made of the ES1 strain presence in all the isolated organs in order to detect their possible translocation. This search was unsuccessful, even in the case of immunosuppressed animals.[30]

Furthermore, the ES1 strain genome was sequenced at Biopolis SL using mass pyrosequencing technology, confirming the molecular absence of genes related to antibiotic resistance, virulence or pathogenicity factors. This study currently provides a complete annotation of the probiotic strain ES1 genome. Surely this fact will help understand the molecular basis of their anti-inflammatory behavior.

6. Clinical Trials

After the above, two ES1 probiotic clinical trials with human volunteers were conducted. Both were coordinated by the IATA-CSIC group. In the first, healthy adults were given probiotic or placebo pills for fifteen days. Subsequently, a two-week washout was performed and the groups were crossed. None of the trial participants expressed discomfort or intestinal problems or of any other type. Furthermore feces of these individuals were analyzed, determining the preponderance of the ES1 strain.

The second trial focused on celiac children who were beginning a gluten-free diet. They were given the probiotic or a placebo for three months. The study was conducted at the Sant Joan de Reus Hospital and the Sant Joan de Déu Hospital at Barcelona. The results indicated that some cell products involved in the inflammatory response are statistically reduced in a significant way in the group receiving the ES1 probiotic. Furthermore, the intestinal flora of the children receiving the probiotic had significant positive changes in relation to the group receiving the placebo, reducing *Bacteroides* and *Clostridium* counts and increasing bifidobacteria. At the time of this writing a scientific paper with all these results is being written.

7. Technological Development of *Proceliac*

Encouraged by these results, researchers from the R & D Department at the Asturian Milk Central developed *Proceliac*. To this end they combined the ES1 probiotic with a number of nutrients important for growth (calcium, iron and vitamins B1 and B5), or for the immune response (selenium, zinc, vitamins A, B6 and B12) in normal or celiac individuals.[31] It should be remarked that, regarding calcium, *Proceliac* provides 50% of the recommended daily dose while only 15% of the other nutrients (Table 1).

The product is a low-fat dehydrated milk that comes in two different formats: a family-sized can with a dispenser that offers 14 servings or a box containing 14 single-dose packages (Figure 2). Currently new product concepts are being developed which, based on these previous developments, improve the offer of these kind of products. For example, the development of this same kind of products, free of lactose and with added cocoa or vanilla is reaching its final stages.

The preceding pages allow us to conclude that *Proceliac* is a dairy product specifically aimed at and designed with celiac patients in mind. It is a new development in the gluten-free diet world by combining a probiotic and being backed by solid scientific experimentation that has produced scientific publications in prestigious journals. It should be noted that, by using it, an improvement in welfare of the celiac community is sought, but of course, this product is not intended to replace the gluten-free diet or to allow voluntary transgressions. Its role is different: to alleviate intestinal inflammation and restore the celiac patient's microbiota.

Important information	Contents of 30g/250 ml glass	% CDR
Energy value (Kcal/Kj)	105.9 / 449.6	
Protein (g)	8.2	
Carbohydrates (g)[1]	17.7	
Lactose (g)	< 0.01	
Glucose+ Galactose (g)	12.8	
Dextrose (g)	4.9	
Fats (g)[2]	0.25	
Of which saturated (g)	0.16	
Sodium (g)	0.13	
Calcium (mg)	400	50.0
Potassium (mg)	400	20.0
Iron (mg)	2.1	15.0
Zinc (mg)	1.5	15.0
Selenium (ug)	8.3	15.0
Vitamin A	120	15.0
Vitamin D	1.0	20.0
Vitamin E	1.8	15.0
Vitamin B1	0.17	15.0
Vitamin B5	0.9	15.0
Vitamin B6	0.21	15.0
Vitamin B12	0.38	15.0

Table 1. Proceliac Nutritional composition ([1] All sugars; [2] Saturated 0.16 g).

Figure 2. Proceliac formats.

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