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Study of biochemical mechanisms induced by Gluten Friendly ™ *in vitro* and *in vivo* on coeliac patients

Ph.D. Student:

dr. Musaico Daniela

Tutor:

dr. Carmela Lamacchia

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"Try not to become a man of success, but rather try to become a man of value."

(Albert Einstein)

To my grandmother Lina, my parents and Antonio…

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Abstract

This thesis aimed to investigate the biochemical mechanisms induced by "Gluten Friendly™" *in vitro* and *in vivo* on coeliac patients.

"Gluten Friendly™" is a patented technology (Italian patented method n°: 0001414717, also filed under the Patent Cooperation Treaty, application no. PCT/IB2013/000797 and published in Europe as EP 2903453 A1 and titled "Detoxification method of gluten proteins from cereal grains") that allows the reshaping of gluten proteins directly in the cereal grains, before milling. Thereby it is possible to obtain functional flours suitable for the preparation of bakery products made from wheat. Specifically, "Gluten Friendly™" technology involves the application of microwave energy for a few seconds to hydrated wheat kernels before milling to reach a high temperature for a short amount of time. The method aims to combine the nutritional, organoleptic and rheological properties of wheat proteins with a beneficial role for the gut health and microbiota of both celiac and healthy subjects. This innovative technology relies on a wise combination of parameters such as temperature, humidity, resting, and evaporation, clearly described in the registered patent and quoted in this thesis. This temperature-based process does not cause gluten proteins denaturation, but changes in their conformation, even in the so-called antigenic sequences, that make them unrecognizable *in vitro* by a wide range of antibodies specific for gluten proteins. It has been postulated that "Gluten Friendly™" technology induces rearrangements of protein structure that involve the exposure of charges that may allow a new kind of aggregation among different classes of wheat endosperm proteins, only through hydrophobic and/or ionic interactions. The investigation of gluten proteins reshaping induced by Gluten Friendly technology requires tough efforts since the Gluten Friendly molecule shows difficulty in its isolation. However, the molecular structure could be investigated by using an approach based on integrated techniques such as Nuclear Magnetic Resonance (NMR) spectroscopy and Infrared (IR) spectroscopy. While the temperature-treated gluten loses its immunogenic properties *in vitro* on celiac, gut-derived T-cells lines, it is able to act as a bioactive molecule with pleiotropic effects on human health, without losing its technological proprieties and viscoelasticity. The flour can still form dough, leaven and bake into bread. The sensory qualities of Gluten Friendly™ bread (GFB) are comparable to that of the Control Bread (CB) in terms of appearance, taste, aroma, colour and bread texture. Furthermore, a previous *in vitro* study carried out on celiac and healthy fecal microbiota pinpointed that GFB prolonged the survival of *L. acidophilus* La-5 and exerted an antibacterial effect towards *S. aureus* and *S. Typhimurium*. GFB was able to positively modulate the composition of celiac gut microbiota *in vitro*, promoting changes in Lactobacilli and Bifidobacteria members. These results were confirmed in a following *in vitro* study, performed by a three-stage continuous gut model system, on the intestinal microbiota composition and metabolites production of healthy and celiac subjects for a prolonged period of 30 days. The study showed the ability of GFB

to restore even the microbiota of healthy individuals, increasing the number of bifidogenic bacteria and SCFAs levels. Based on this evidence, the technology has been further improved with the registration of a second patent: "Method for the detoxification of gluten proteins from cereal grains and related uses in the medical field" (Italian priority patent no: 102015000084813 also extended to European level PCT/EP2016/0815899).

Further *in vitro* study showed that GFB, in comparison to CB, increased mucus secretion and gut-barrier function in human intestinal goblet cells. These data support the potential effect of GFB to maintain or improve gut homeostasis and cellular barrier integrity by maintaining higher mucin levels. Moreover, another study based on the preparation of synbiotic fermented milk, containing *L. acidophilus* La-5 and Gluten Friendly^{TM} flour (GFF), was carried out. The most important result was the positive effect of GFF on the viability of the probiotic, with a prolongation of the shoulder length to 20 days (12–13 days in the control). Based on this evidence, another *in vitro* study, aiming to produce a synbiotic yogurt containing *Bifidobacterium infantis* and GFF, was carried out. The results highlighted that GFF prolonged the viability of Bifidobacteria for 14 days instead of 4 days in the control and control flour (CF). Based on the reduced immunoreactivity and the beneficial effects of Gluten Friendly^{TM} molecule on the gut microbiota *in vitro*, an *in vivo* prospective, double-blind, randomized, placebo-controlled study of 12 weeks was performed on 48 celiac volunteers. After 12 weeks on a Gluten Friendly^{TM} diet, it induced i) symptoms relief in all patients; ii) a clustering of the mucosal histology and inflammation of celiac subjects in 3-zones, as follows: 35% black zone (cluster 1; increase of IELs and injury), 30% grey zone (cluster 2; intermediate trend), 35% white zone (cluster 3; normal serology, no injury and no increase of IELs, trend as the placebo group). Thus, suggesting positive effect in 35% of patients (white zone) and a possible dynamic situation, with a positive modulation of the inflammation, in 30% of patients (grey zone). iii) a shaping of celiac microbiota towards intestinal homeostasis through a bifidogenic effect that Gluten Friendly induced by its digestion on Lactobacilli and other microorganism butyrate-producing species that contribute to the gut " health ecology" of celiac subjects.

Aim and outlines of the thesis

This thesis is aimed to understand the biochemical mechanisms induced by the tertiary structure modification of the Gluten Friendly^{TM} molecule *in vitro* and *in vivo* on celiac subjects. Following the general introduction in *Chapter 1*, *Chapter 2* describes the *in vitro* effects of digested GFB on the intestinal epithelium mucus production and barrier function in healthy human mucus-secreting goblet cells HT-29 16E. Mucus production was investigated using staining techniques and ELISA assay, instead, the barrier function was evaluated by trans-epithelial electrical resistance (TEER) measurements. Based on the positive modulation exerted *in vitro* by the GFB on the gut microbiota

composition, *Chapter 3* describes the impact of the GFF supplementation on the functionality of an active drink. In this study, the optimization and realization phases of synbiotic fermented milk, containing *L. acidophilus* La-5 and GFF were carried out. The optimization of the production of the active drink was performed through a simplex centroid design in order to study the effects of the variables such as flour, temperature and probiotic on the acidification. Thus, the following conditions were chosen to produce the fermented milk: *L. acidophilus* at 6.5 log cfu/ml; flour at 2.5 g/l; temperature at 37°C. Then, the fermented milk was produced and stored at 4°C for 90 days. Finally, the impact of GFF on the viability of the probiotic was evaluated.

Chapter 4 aims to evaluate the *in vivo* effects and safety of the GFB on celiac patients. In particular, GFB was tested through a prospective, double-blind, randomized, placebo-controlled study of 12 weeks on 48 celiac patients. Serological levels of EMA, anti-tTGA, and pro-inflammatory cytokines were analyzed at the baseline, after a month and at the end of the study. The histological analysis on duodenal biopsies (Vh/Cd decrease and IELs increase), and gut permeability were carried out at the baseline and after 12 weeks. Stool samples were analyzed on a monthly basis throughout the 12 weeks of GFB daily intake by G-12 ELISA assay. Symptoms were analyzed weekly by the CSI system.

In the final analysis, *Chapter 5* consists of the general discussion on the obtained results from *in vitro* and *in vivo* studies, conclusions, and future investigations.

Chapter 1

General Introduction

1.Wheat

Botanically, wheat and other cereals (e.g., rice, barley and maize) belong to the grass family *Gramineae* which, with about 10.000 species, represents one of the largest families of flowering plants [1]. Cereals such as maize, rice, wheat, sorghum, millet, barley, and oats are particularly important as a staple food all over the world [2]. In particular, wheat (*Triticum spp*.), consumed by billions of people, is the major staple food in many diets, providing a large proportion of the daily energy intake. In 2016, the global production of wheat exceeded 749 million tonnes, making it the second most-cropped cereal after maize [3]. The cultivation of wheat reaches far back into history. Wheat was one of the first domesticated food crops and its first cultivation occurred about 10.000 years ago, as part of the 'Neolithic Revolution', which saw a transition from hunting and gathering of food to settled agriculture. Cultivation spread to the Near East: the center of origin of cultivated wheat was the Fertile Crescent, a region extending from south-western Iran, through the Tigris and Euphrates basins in northern Iraq and south-eastern Turkey extending to central Israel and Jordan. The main route into Europe was via Anatolia to Greece and across to Italy, France, and Spain reaching the UK by about 5000 years ago. Similarly, the wheat spread reached China and Africa and finally, it was introduced by the Spaniards to Mexico and Australia [5].

The earliest cultivated forms of wheat were essentially landraces selected by farmers from wild populations, presumably because of their superior yield and other characteristics. However, domestication was also associated with the selection of genetic traits that separated them from their wild relatives [4, 6]. These cultivated forms were diploid kind of wheat such as einkorn (genome AA with two complements of seven chromosomes, 2n=14) and tetraploid such as emmer (two closely related subgenomes AABB). In particular, einkorn wheat (one-grained wheat), *Triticum monococcum*, is believed to be the most ancient cultivated species of wheat and maybe the species from which all cultivated wheat is descended [1, 4].

Nowadays, cultivated wheat is classified into three main groups: diploids (einkorn), tetraploids (emmer, durum, rivet, Polish and Persian wheat), and hexaploids (spelt, bread, club and Indian shot wheat). Most tetraploid wheats are derived from wild emmer (*Triticum turgidum*) which is itself the result of a hybridization between two diploid wild kinds of grass that occurred in the wild, long before domestication, driven by natural selection. Either domesticated tetraploid emmer or durum wheat (*T. turgidum sp. durum*) hybridized with yet another wild diploid grass, *Aegilops tauschii* (genome DD), to make the allohexaploid wheats. These wheats have three closely relate subgenomes (A, B and D), three times as many as in diploid wheat. Therefore allohexaploid wheat genomes are considered as a multi-level phylogenetic mosaic, although a clear understanding of their phylogenetic history has been lacking. [7, 9, 11, 12]. Only in the second half of this century, it became clear that there are no

wild hexaploid progenitors and that cultivated hexaploid wheats were formed in farmers' fields by hybridization between cultivated tetraploid wheat and wild diploid species. Nevertheless, the botanical classification of wheat varieties still remains not unified [6,7].

Overall, hybridization, domestication and strong selection pressure had reduced the level of genetic diversity available to wheat breeders, and this lack of diversity was widely recognized as a limiting factor in the breeding of high yielding varieties. For this reason, modern species have been extensively modified and subject to cross-breeding in what is commonly referred to as the "Green Revolution". The principal results of this revolution were the development of modern varieties characterized by higher yield, reduced susceptibility to diseases and insects, increased tolerance to environmental stresses, a homogeneous maturation (to optimize harvest) and a higher gluten content (to improve bread and pasta quality) [3,8,12]. Furthermore, increasing wheat yield is a major global priority for feeding the world's growing population. It has been estimated that wheat yields need to increase by 50% by 2050 to meet this demand. Thus, in recent years, wheat genetic manipulation has paved the way to design new strategies for the improvement of complex crop species such as polyploid wheats. The genetic constitution of wheat is important because all quality traits result from the expression of genes and their interaction with the environment. The integrated use of omics technologies and analysis of omics-based data sets have facilitated numerous studies in a plant system. The omics methodologies have been applied first for DNA analysis (genomics), then extended for RNA (transcriptomics), proteins (proteomics), and small metabolites (metabolomics). These technologies significantly contribute toward the identification of important genes and their manipulation for cereal improvement [11, 12, 14].

Today, about 95% of the wheat produced is *Triticum aestivum*, a hexaploid species usually called "common", "bread" or "soft" wheat. *Triticum aestivum* is the "king of cereals" and a great number of *aestivum* wheat varieties are grown in the world. The remaining 5% is primarily comprised of *Triticum durum* or "durum" wheat, a tetraploid species predominantly used for making pasta. Other wheat species (e.g., spelt wheat) are grown in limited quantities, mostly for specialized use [7, 15]. Wheat is grown on more land area than any other commercial crop and continues to be the most important food grain source for humans. Within a species, wheat varieties can be further classified by growing season (winter vs. spring wheat), the hardness of the grain (hard vs. soft wheat), and the color of the grain (red, white or amber). According to these criteria, all wheat species fall into one of the six major classes (classification system used in the USA): Hard Red Winter, Durum wheat, Hard Red Spring, Hard White, Soft Red Winter and Soft White [9]. The classification into spring or winter wheat is common and traditionally refers to the season during which the crop is grown. Winter wheat varieties are sown late September to November (they lie dormant during the winter) and harvested

early in the summer. Whereas spring wheat varieties are sown February to April and harvested late in the summer. The optimum growing temperature is about 25°C, with minimum and maximum growth temperatures of 3° to 4°C and 30° to 32°C, respectively. Optimal production requires an adequate source of moisture availability during the growing season; however, too much precipitation can lead to yield losses from disease and root problems [5]. Hard wheat varieties have higher gluten content (10% to 14% proteins) than soft wheat grains (8% to 11% proteins), and their flours are preferred for leavening. *Durum* wheat has protein levels varying from 9% to 18% and is the hardest of all wheat species. *Durum* wheat flour is used for macaroni, spaghetti, and other pasta products. The soft wheat grains have a higher starch-to-protein ratio compared to hard wheat varieties. In general, unlike flour from hard wheat flours, which is mainly used for bread making, soft wheat flour is used for pastry [9,10]. With regards to the color of the grains, it is known that many wheat varieties are reddish-brown due to phenolic compounds present in the bran layer which are transformed to pigments by browning enzymes. White wheats have a lower content of phenolics and browning enzymes and are generally less astringent in taste than red wheats. The amber color of durum wheat is due to a carotenoid pigment called lutein, which can be oxidized to a colorless form by enzymes present in the grain [9].

1.2 Wheat grain structure

Wheat-based products are important staple foods for several billions of people in more than 100 countries. Within the context of a balanced diet, wheat represents a healthy source of multiple nutrients, dietary fiber, and bioactive compounds, especially if consumed as a whole-grain [3, 15].
The grain is the fruit of the wheat, botanically called caryopsis [9, 16]. Wheat grains are generally oval-shaped with a bundle of hairs, which is referred to as the beard or brush, at the top. They have a length between 5 and 9 mm, weighs between 35 and 50 mg and a crease down one side where they were originally connected to the wheat flower. The wheat grain (Fig.1.1) contains 13-17% bran, 80-85% mealy endosperm and 2-3% germ (all constituents converted to a dry matter). It consists of three distinct parts: bran, endosperm and germ. The bran (outer coating of wheat grain) is made up of several layers, which protect the main part of the grain from weather, insects, soil, and bacteria. It is separated from the starchy endosperm during the first stage of milling. Bran is rich in B vitamins, minerals (7.2%) and water-insoluble fibers (53%). Chemical composition of wheat bran fiber is complex, but it contains, essentially, cellulose and pentosans which are typical polymers present in the cell walls of wheat. The two external layers of the grain (pericarp and seed coat) are made up of empty cells and are dead. Proteins and carbohydrates represent roughly 16% of the total dry matter of bran. The endosperm is the largest and most important part of the grain. The outer endosperm, the

aleurone layer, has a different structure: it consists of single layer of cuboid-shaped cells with a thick cell walls, large nuclei and dense cytoplasm. The aleurone layer is rich in proteins and enzymes, which play a vital role in the germination process. Subaleurone cells, which are peripheral endosperm cells, constitute one cell layer, made up of cells that are smaller than the inner endosperm cells. Below the subaleurone layer, the inner endosperm, with its prismatic and highly-vacuolated cells, is referred to as mealy or starchy endosperm. The endosperm mainly contains food reserves, which are needed for the growth of the seedling; it is rich in energy-yielding starch granules. The starch is distributed fairly evenly throughout this tissue, with the exception of the sub-aleurone cells where the content of starch is significantly lower. No starch is present in the cells of the aleurone layer. Apart from carbohydrates (82%), the mealy endosperm contains fats (1,5%) and proteins (13%): albumins, globulins and the proteins that will form the gluten complex at dough making, glutenins and gliadins. Instead, the contents of minerals (ash) and dietary fibers are low. Finally, the germ is the embryo of the seed and lies at one end of the grain. It contains proteins, minerals and is rich in lipids (8-13%). The germ is present in whole-wheat flour but is usually separated from the flour during the milling process because of fat content that limits the flour quality and shelf-life. Furthermore, wheat germ is available as a separate entity because it is an important source of vitamin E [16, 17, 59].

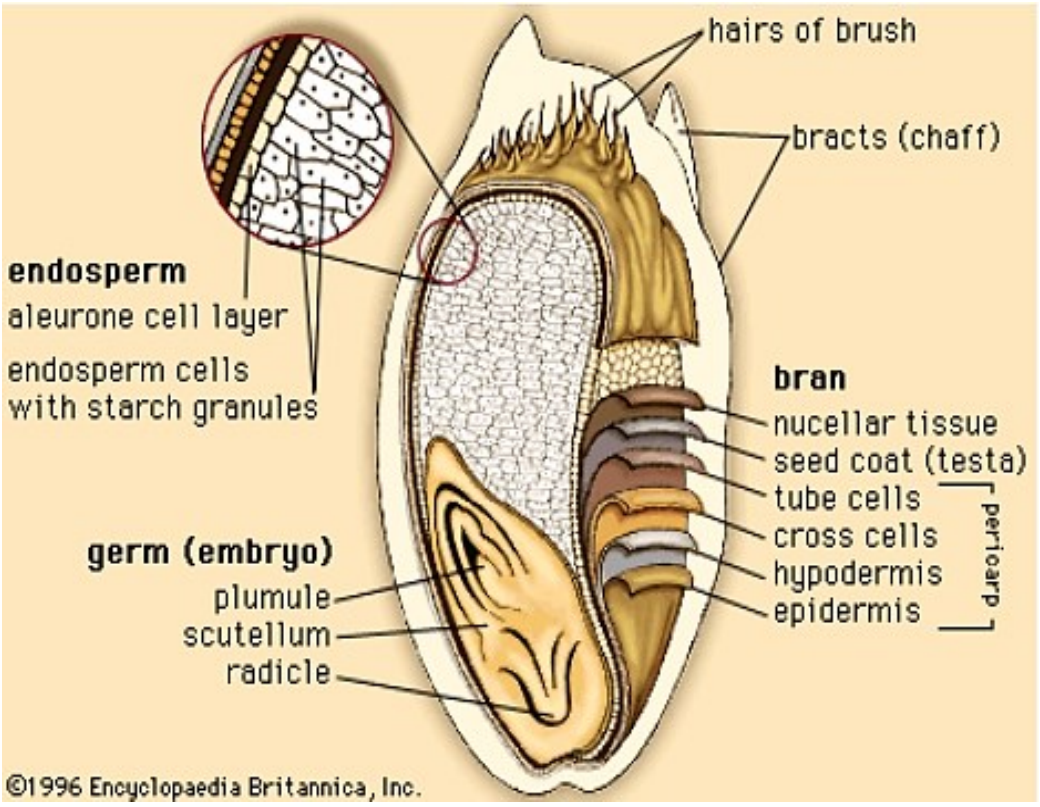


Fig. 1.1. Structural diagram of the wheat grain cut lengthwise [16].

1.3 Wheat proteins

Plant seeds contain compounds stored as sources of energy for supporting germination. Profiles of the carbohydrates, proteins, and lipids reflect the specific requirements for seed and seedling survival [8]. Wheat is unique because its flour can form a dough with properties required for the production and the quality of a wide range of baked products. Although starch is quantitatively the major constituent of the wheat grain, the unique properties of the kernel reside primarily in the gluten-forming storage proteins of its endosperm. For most traditional uses, wheat quality derives mainly from grain hardness and protein content, with each end-use requiring a particular protein quality. Quality is determined by the molecular structure of the storage proteins of wheat, which are essential during the bread-making process [10,13, 17]. The protein content of mature wheat grains generally may vary between 10% - 18% of the total dry matter. Proteins are unevenly distributed in the grain and their content is determined by genetic and environmental factors, such as the presence of "high protein genes" or the availability of nitrogen fertilization [20].

Wheat proteins are classified into four groups according to their extractability and solubility in different solvents. Classification is based on the classic work of T.D. Osborne at the beginning of the last century. Sequential extraction from the milled grain results in the following protein fractions: albumins (water-soluble proteins), globulins (insoluble in pure water, but soluble in dilute NaCl solutions), gliadins (soluble in alcohol-water solutions usually 60-70% (v/v) ethanol or 50% (v/v) propan-1-ol) and glutenins (soluble in dilute acid or sodium hydroxide solutions, detergents or alcohol/water mixtures in which a reducing agent, such as dithiothreitol or 2-mercaptoethanol, is included to reduce inter-chain disulfide bonds). Each of the mentioned fractions is a complex mixture of different proteins, which overlap in their solubility. The separation of albumins and globulins turned out to be not as clear as initially suggested by Osborne, as well as the separation of the gliadin and glutenin fractions [16, 17, 35]. Thus, all four fractions can be contaminated by any proteins from other fractions. At present, many slightly modified versions of this procedure exist and are used to isolate the different wheat protein fractions. Alternatively, wheat proteins are classified into the non gluten-forming proteins on the one hand and the proteins that form the gluten network, on the other hand. The non gluten-forming proteins, which generally enclose approximately 15% to 20% of total wheat proteins, correspond to the albumins and globulins in Osborne type fractionation procedures. The albumin and globulin proteins are less important to bread-making quality than the gluten proteins. Conversely, the gluten proteins consist of gliadins and glutenins, which have very low extractability in water or salt solutions due to their primary structure. In fact, these proteins are rich in proline and glutamine residues and are usually called prolamins. Gliadins and glutenins together represent about 80% of the total proteins in flour and are present in

approximately equal amounts. Prolamins, deposited in discrete protein bodies in the development starchy endosperm, are the major storage proteins in most cereal seeds and the most important determinants of the functional properties of wheat flour. Ideal dough properties depend on an appropriate balance between gliadin (contributing to dough viscosity) and glutenin (contributing to the strength and elasticity of dough). It is the unique combination of these proteins that comprises the functional properties of dough [4, 10, 21-23]. Furthermore, the wheat proteins are also classified based on their biological, chemical or genetic characteristics, rather than solubility. Nevertheless, Osborne`s classification system was a major milestone in cereal chemistry. In fact, this classification is still in use, despite the number of factors, such as fineness of grain milling, vigor and time of shaking, the concentration of extraction solutions used, and the extraction temperature, which influence the extractability of particular protein fraction [19].

1.3.1 Albumins and globulins

In addition to storage proteins, wheat kernels contain also albumins and globulins which are soluble proteins easily extracted from flour using a water and sodium chloride solution. However, the separation of albumins and globulins turned out to be not as clear as initially suggested by Osborne. These proteins are concentrated in the seed coats, the aleurone cells, and the germ, with a lower concentration in the mealy endosperm. The albumin and globulin fraction cover about 20% - 25% of the total kernel proteins [19, 27]. Albumins are the smallest wheat proteins, followed in size by globulins. Both protein classes are mainly monomeric with a molecular weight (MW) pattern ranging from 2 to 106 kDaltons (Da), predominantly divided into two relatively wide regions with MW from 23 to 66 kDa and 2 to 16 kDa, definitely lower than gliadins and glutenins. Furthermore, albumins and globulins tend to form polymers stabilized by inter-chain disulfide bonds. Most of the physiologically active proteins (enzymes) in wheat grains are found in the albumin and globulin groups. In fact, these proteins are not involved in the gluten formation, but only in metabolic processes (e.g., starch synthesis and degradation) as the enzymes (e.g., α- and β-amylases) or enzyme inhibitors (e.g., α-amylase inhibitor), and minority of albumins and globulins are structural proteins [16, 17, 19, 27]. Nutritionally, the albumins and globulins have a very good amino acid balance: they are relatively rich in lysine, tryptophan and methionine and contribute about 50% of the total lysine content in the grain proteins [19]. Among the varieties, both albumins and globulins show very similar protein pattern and, consequently, these proteins are not suitable for wheat varieties identification.
Considering the general opinion that albumins and globulins of wheat endosperm have a secondary role in influencing the flour quality, they have been insufficiently studied. However, there is evidence

in the scientific literature for the contribution of non-gluten proteins to the biochemical and rheological properties of the dough. Furthermore, the albumin and globulin fractions may be relevant for normal baking properties even if they do not play a direct role in the bread-making process [28, 29].

1.3.2 Gluten

Among the cereal grains, wheat is unique because wheat flour can form a dough that exhibits the rheological properties required for the production of leavened bread and the wider diversity of baked foods. The unique properties of the wheat grain reside primarily in the gluten-forming storage proteins of its endosperm, which normally are digested during germination to provide nutrients (carbon, nitrogen and sulfur) for the growing seedling [13, 20, 34]. Gluten comprises about 75-85% protein on a dry weight basis, with most of the remainder being starch, non-starch carbohydrates and lipids. The gluten proteins have been widely studied over a period of 250 years, in order to determine their structures and properties and to provide a basis for manipulating and improving the end-use quality. For this reason, the volume of wheat gluten research is wide and reflects the complexity of the gluten system which remains incompletely understood. Gluten was one of the earliest protein fractions described by chemist Beccari in 1728. It is defined as a cohesive, visco-elastic proteinaceous complex that remains when the dough is washed to remove starch granules and other components soluble in water and dilute saline solutions [4, 21, 30, 34]. This resulting proteinaceous bulk retains its cohesiveness on stretching (Fig.1.2) and plays a key role in determining the rheological dough properties. The gluten protein network varies because of different components and sizes, and variability is caused by genotype, growing conditions, and technological processes. Genetic studies have investigated the polymorphisms which exist among the gluten protein fractions, present in different genotypes, in order to establish genetic linkages between either group of gluten proteins and aspects of processing quality. Similarly, biochemical and biophysical studies have demonstrated a relationship between dough strength and the ability of the gluten proteins to form polymeric complexes. The structures and interactions of proteins contribute to the unique properties of gluten and are essential for the dough quality of bread and other baked products such as pasta, cakes, pastries and biscuits [4, 10, 31]. Wheat gluten has the capacity to act as a binding and extending agent, thus is commonly used as an additive in processed foods for modifying the structure and improving texture, flavor and moisture retention. Of note, in order to meet the consumer needs, food industries have made in the last fifty years an indiscriminate use of wheat gluten even in food that is naturally devoid [38]. It is present in processed meat, vegetarian meat substitutes or as thickener, emulsifier or gelling agent in candies, ice creams, butter, seasonings and as filler and coating in medications or

confectionary [18, 31]. Probably the increased use of gluten contributes as an environmental factor in the onset of celiac disease (CD) in genetically-predisposed individuals [31, 32].

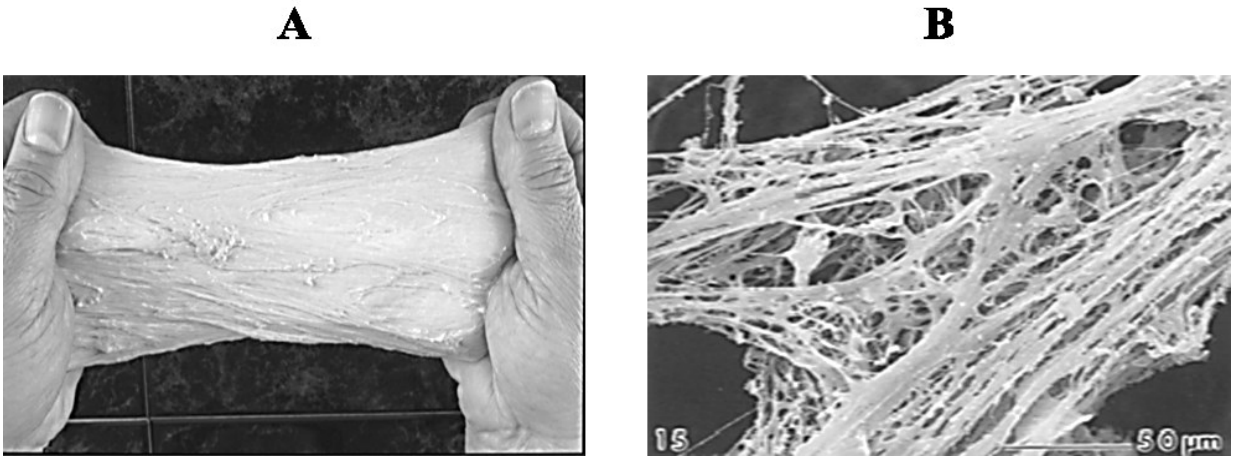


Fig. 1.2. Representative images of gluten network: **(A)** the cohesive properties of gluten when it is stretched and **(B)** how gluten proteins begin to stretch and bond together after kneading; (modified from [4]).

Properly, gluten is a mixture of hundreds of related but distinct proteins, varying in isoelectric point (pI) and MW. It is formed by monomeric gliadins and polymeric glutenins respectively, also called prolamins for the high contents of glutamine and proline (with 15% and 36% on average respectively) and low contents of charged amino acids. Besides proline and glutamine, cysteine is another extremely important amino acid for the structure and functionality of gluten, even though it belongs to the minor amino acids (~2%) of gluten proteins. Cysteine residues are mostly present in oxidized state and form either intra-chain disulfide bonds within a single protein (both gliadins and glutenins) or inter-chain disulfide bonds between glutenins [24, 30]. The reduction of these inter-chain bonds allows the separation of the glutenin subunits into low molecular weight (LMW) and high molecular weight (HMW) groups. Both intra- and inter-chain disulfide bonds, as well as non-covalent interactions (hydrogen, hydrophobic, ionic, van der Waals') are involved in the formation of gluten complex [23, 34, 39]. Recently a new system of classification, based on the complete amino acids sequences and structural and evolutionary relationships, divided the gluten proteins into three groups (Fig.1.3): sulphur-rich (S-rich), sulphur-poor (S-poor), and HMW prolamins [30, 38, 39]. Sulphur-rich group includes α/β-, γ- gliadins and B- and C-type LMW glutenins subunits (LMW-GS); sulphur-poor prolamins group includes the monomeric ω-gliadins and the D-type LMW-GS; and finally, the HMW prolamins group include the HMW glutenins [24, 35, 38, 39].

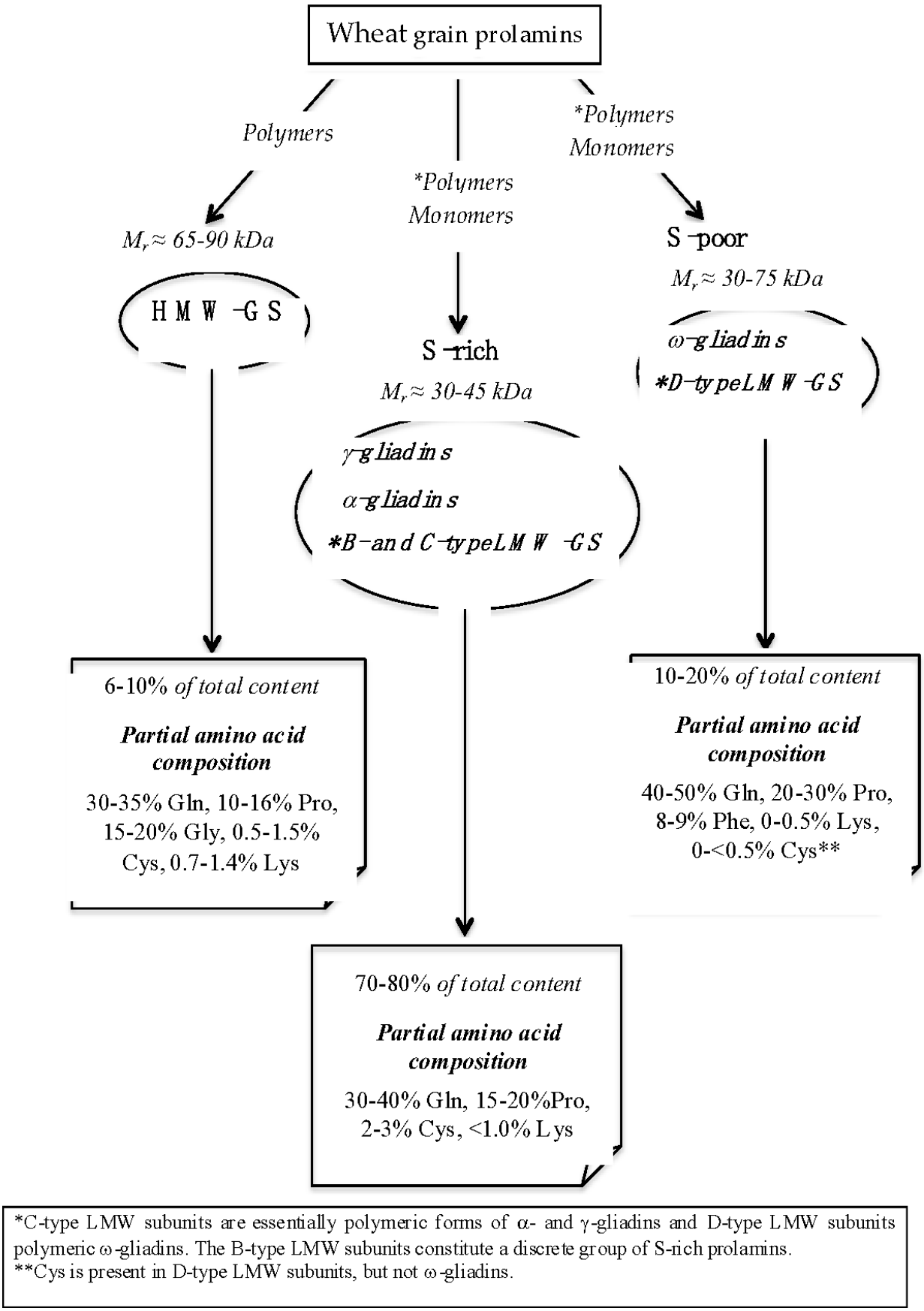


Fig. 1.3. Schematic representation of types and peculiarities of wheat gluten proteins (gliadins and glutenins) [38].

In particular, components related to ω-gliadins are also present in the glutenin polymers. These components, previously defined as the D of LMW-GS, are now known to be the mutant form of ω-gliadins, in which mutation has led to the presence of single cysteine residues, and hence the ability to became incorporated into polymers by inter-chain disulfide bonds [35].

Most of the interest in prolamins is related not to their nutritional quality, but to their impact on grain processing proprieties [26, 32-36]. Both gliadins and glutenin have no enzyme activity, but are unique because are technologically active though their functions are divergent [16, 17, 38]. Hydrated gliadins are less cohesive and have little elasticity than glutenins, and contribute mainly to the viscosity and extensibility of the dough. Contrarily, hydrated glutenins are both cohesive and elastic and are responsible for dough strength and elasticity (Fig. 1.4) [38, 40, 41]. In fact, it is well established that strong doughs (i.e., highly visco-elastic) contain high proportions of HMW glutenin polymers. For this reason, numerous studies suggest that the HMW subunits form an elastomeric polymer network which provides a "backbone" for interactions with other glutenin subunits and with gliadins (Fig. 1.5) [38, 43, 44, 46]. Obviously, three-dimensional gluten network is mainly stabilized by inter-chain disulfide bonds, but additional covalent bonds are tyrosine-tyrosine and thiyl-tyrosine crosslinks between gluten proteins, formed during dough making [47]. However, the covalent structure of the gluten network is superimposed by non-covalent bonds [38, 39, 45]. Though this class of chemical bonds is less energetic than covalent bonds, they are clearly implicated in gluten protein aggregation and dough structure [30]. Gluten proteins are susceptible to heat treatments, and their behavior under relatively high temperatures has been investigated. When glutenins are heated above 55°C and gliadins above 70°C, disulfide (S-S) / sulphydryl (SH) exchange reactions occur, showing that heating particularly influenced the S-S structure of gluten proteins [53].

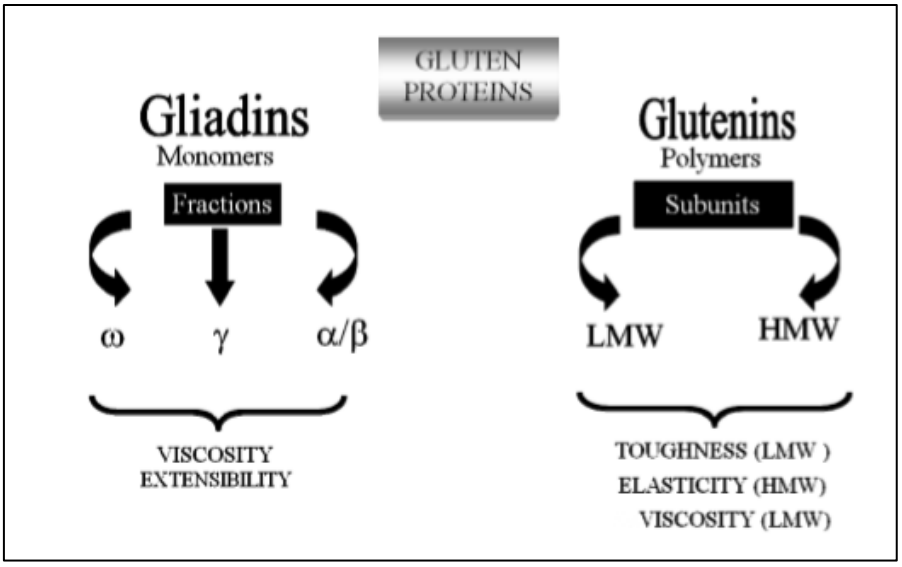


Fig. 1.4. Gluten proteins: fractions and technological properties [38].

Wheat prolamins are the major storage proteins present in the wheat starchy endosperm, where they are synthesized, folded and deposited via the endomembrane secretory system. Like all proteins destined for the secretory pathway, gluten proteins are synthesized on polyribosomes attached to the Rough Endoplasmatic Reticulum (RER) and pass via the usual translocation machinery into the lumen, with the loss of an N-terminal signal peptide cleaved by signal peptidase. This signal sequence is therefore not present in the mature protein. Once within the lumen, protein folding and disulfide bond formation, as well as the formation of the inter-chain bonds which stabilize the glutenin polymers, occur with no further post-translational modifications taking place (i.e. no glycosylation or proteolysis as may occur with other types of seed storage proteins). The folding and assembly of the gluten proteins are probably assisted by ER lumenal proteins (i.e. molecular chaperones or protein disulfide isomerase) although this is still not conclusively established [4, 29, 37]. The subsequent fate of the prolamins is also unclear, it may vary with the protein type, with the age and stage of the tissue development. Electron microscopy evidence indicates that two separate pathways may operate in the developing wheat grain. In fact, some of the proteins appear to be transported via the Golgi apparatus in their route from the ER to the vacuoles, where they form protein bodies. Instead, others are accumulated directly within the lumen of the ER to form a second population of protein deposits. The individual protein bodies range in diameter up to about 20 μm. Gliadins are preferentially transported to the vacuole via the Golgi apparatus, conversely, glutenins are preferentially retained in the ER. It is not known why some gluten proteins accumulate within the ER while others pass via the Golgi to the vacuole. It has therefore been suggested that accumulation in the ER lumen could be a consequence of the ability of glutenins to form insoluble aggregates and rapid assembly into HMW polymers, which precipitate and accumulate directly within the ER lumen. Conversely, monomeric gliadins, which are soluble in the lumen environment, are readily transported to the Golgi [4, 34, 37, 39, 42].

Moreover, clear gradients exist in protein bodies concentration across the starchy endosperm. Cells derived from the subaleurone layer may contain up to 45% protein, mainly gluten protein, in contrast to the central endosperm cells in which protein may only account for about 8%. The protein gradient is not only quantitative but also qualitative, in that different sub-classes of gluten proteins are differentially expressed in different regions of the endosperm. Immunoflorescence evidence suggests that cells of the central starchy endosperm clearly contain some protein bodies with HMW subunits of glutenin but little or no gliadins. Instead, LMW glutenin subunits and gliadins appear co-localized in the same protein bodies, predominantly in the cells of the subaleurone layer [37, 59, 60]. As a result of this process, individual cells of wheat kernel contain a quali-quantitative gradient of protein bodies, which are brought together when flour is mixed with water to form a continuous, visco-elastic

network in the dough with unique rheological properties, capable of retaining gas bubbles [16, 38]. It is understood that wheat gluten owes its unique visco-elastic behavior to an appropriate balance in the amounts of gliadin and glutenin proteins, thus variation in their number and composition appear to be largely responsible for the differences in the gluten visco-elasticity among wheat varieties [25].

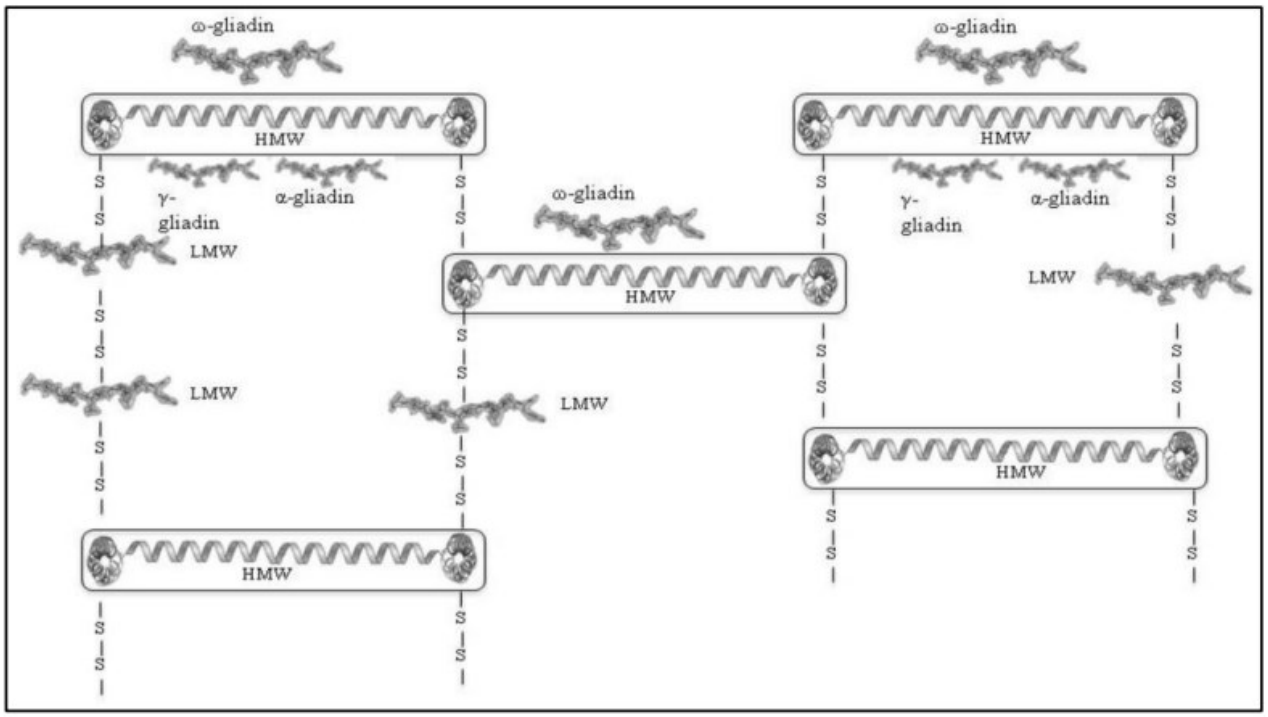


Fig. 1.5. A structural model for wheat gluten in which the HMW subunits provide a disulfide-bonded backbone that interacts with other gluten proteins by disulfide bonds (LMW subunits) and non-covalent interactions (gliadins); (modified from [44]).

1.3.3 Gliadins

Gliadins constitute from 30 to 40% of total flour proteins and represent large families of monomeric wheat proteins. They are particularly important in food science because they contribute to the viscosity of the gluten network, which results predominantly from non covalent interactions, as hydrogen and hydrophobic bonds between the individual gliadin monomers and glutenin polymers [19, 26, 44]. Closely related proteins to the gliadin of wheat exist as secalin in rye, hordein in barley and avenins in oats. Wheat gliadins represent the fraction soluble in alcohol-water mixture, usually 60-70% (v/v) ethanol or 50% (v/v) propan-1-ol [35, 38]. Gliadins were initially classified into four groups based on their gel electrophoresis mobility at acidic pH: α-, β-, γ-, and ω-gliadins (in decreasing order of mobility and increasing order of relative molecular mass) (Fig. 1.6) [25, 30, 33,

48]. Later studies on the amino acid composition showed that the electrophoretic mobility did not always reflect the protein sequence relationships [30]. Consequently, the α- and β- gliadins, which are structurally closely related polypeptides, fall into one group: α/β-type [21, 25, 30]. Thus, three structurally distinct groups of gliadins can be distinguished: α/β-, γ-, and ω-types [10, 26, 37]. Besides, based on the analysis of amino acid sequences and MWs, gliadins could be also divided into four different types: α/β-, γ-, ω1,2- and ω5-gliadins. Within each type, there are small structural differences due to insertion, deletion or substitution of single amino acid, responsible for the protein heterogeneity [30, 32].

The primary structure of gliadins generally consists of several domains of variable sizes. The short N-terminal domain consists of 5 to 14 amino acid residues. The central repetitive domain contains up to 100 residues, organized in repeat sequences of one or two motifs rich in glutamine, proline, and hydrophobic amino acids such as phenylalanine or tyrosine. Finally, the C-terminal non repetitive domain consists of a succession of polyglutamine and unique lysine and arginine-rich sequences that include all the sulfur-containing amino acids [10]. Nevertheless, each gliadin subgroup has different and unique N- and C- terminal domains [26]. In particular, the α/β-gliadins usually contain an N-terminal region of 5 residues, a repetitive domain of 110–130 residues, and a C-terminal region of 140–160 residues [39, 48, 49]. The C-terminal domain is distinguished by a cysteine-rich region that contains four cysteine residues, a glutamine-rich sequence and a region made up of 35–39 residues whose the final two are cysteines. These total six cysteine residues form three intra-chain disulfide bonds that stabilize the folded conformation of gliadins. The MWs of α/β -gliadins ranging from 30 to 34 kDa, due to variations in the lengths of the repetitive domain and the polyglutamine stretches. Instead, the γ-gliadins contain an N-terminal region of 12 residues, a repetitive domain of 80–160 residues, and a C-terminal region of 140–150 amino acids. The C-terminal domain shows a cysteine-rich region with eight cysteine residues, a glutamine-rich region and a final sequence of 41– 43 residues. The eigh cysteine residues are located at highly conserved positions and are all involved in the formation of four intra-chain disulfide bonds. The MW of γ-gliadins ranges from 26 to 46 kDa, but α/β-type and γ-gliadins have generally overlapped MWs, ranging roughly between 30- 46 kDa. Finally, the ω-gliadins contain an N-terminal region of 11 residues, a repetitive domain of approximately 238 residues, and a C-terminal region of 12 residues [10, 48]. These gliadins lack cysteine residues and their primary structure is almost entirely characterized by octapeptide repetitive motif (PQQPFPQQ) rich in glutamine, proline and phenylalanine which together account approximately 80 % of the total amino acid composition [10, 24, 30]. Exactly, in the ω-gliadins the content of glutamine (50%) is higher than proline (20%) [35]. Their relative MWs range between 44

- 80 kDa, with the ω5-gliadins showing higher MW (~50 kDa) than the ω1, 2-type (~ 40 kDa) [22, 23, 25, 30].

The α/β- and γ-gliadins are relatively rich in leucine and tyrosine, but show a lower proportion of glutamine and proline compared to ω-gliadins, although the contents still remains high [10, 23].

The extractability of gliadins from bread is lower than that of gliadins from flour, and α- and γ-gliadins are more affected than ω-gliadins. The effect has been ascribed to sulphydryl (SH)-disulfide (S-S) interchange reactions induced by heat, which affect all gluten proteins, except cysteine-free ω-gliadins [53]. Some gliadins have an odd number of cysteine residues due to point mutations. Those gliadins are linked together or to glutenins and form oligomers with MWs ranging around 100-500 kDa. Such oligomers are called HMW-gliadins. These complexes are solely formed by α/β-, γ-gliadins and LMW glutenins. The total number and relative positions of cysteine residues are important for the size, folding and different behavior of gliadin subunits [22, 25, 30, 39]. Although the gliadin composition is characteristic to variety (genotyping) and growing conditions, it can be generalized that α/β- and γ-gliadins are the major components, whereas the ω-gliadins occur in much lower proportions of wheat varieties [10, 23, 26].

Studies of genetic crosses reveal that the α-and β-gliadins are controlled by Gli-A2, Gli-B2, and Gli-D2 genes on the short arms of the group 2 chromosomes. There are from 25 to 150 copies of the genes that encode the α-gliadins, depending on the variety. Instead, the γ- and ω-gliadins are controlled by clusters of genes (Gli-A1, Gli-Bl, and Gli-Dl) located on the short arms of the group 1 chromosomes. Besides, these gliadins are encoded by clusters of gene families composed of 15–40 and 15–18 copies, respectively. This must indicate a different repeated structure. [23, 48]. With concern to the secondary structure of gliadins, limited structural studies are present in the scientific literature. However, some of them show that the amount of the α-helix structure in the α/β- and γ-gliadins are similar. In particular, the α -helix is mainly present in the non repetitive C-terminal domain, while the N-terminal region contains regularly repeated β-turns, giving to them an overall compact globular structure. In contrast, the β-sheet structure is more present in the γ-gliadins than in the α-gliadins. The secondary structure of the ω-gliadins is quite different from those of the α- and γ-gliadins: it is rich in β-turns and apparently poor in α-helix or β-sheet [10, 21, 23, 26, 48]. Details of the tertiary structure of gliadins are uncertain, as traditional techniques, such as X-ray crystallography and so on, have been unsuccessful with these proteins. Information acquired from other types of analyses has been conflicting. Generally, results are consistent with the conclusion that gliadins are globular proteins with unusual structures composed of multiple disordered regions, rather than a compact structure [50, 51].

1.3.4 Glutenins

The glutenin fraction, which accounts roughly 45% of the total seed proteins, is formed by polymers linked together by inter-chain disulfide bonds, responsible for the giant size of glutenin polymers. In fact, MWs of aggregated glutenin subunits ranging from about 50 to more than 10 million kDa [13, 19, 25]. The increasing of molecular size of glutenin aggregates is linked to the decreasing in their extractability [53]. In addition glutenins, like gliadins, also contain intra-chain disulfide bonds. Native glutenins are insoluble in water or dilute salt solutions, and soluble in acid or alkali, detergents or alcohol/water mixtures in which a reducing agent (usually dithiothrietol or 2-mercaptoethanol) is included to reduce inter-chain disulfide bonds. However, after the reduction of disulfide bonds, individual glutenin subunits are soluble in aqueous alcohols analogous to gliadins [19, 35]. After the inter-molecular disulfide bonds reduction, the glutenin components are broadly subdivided into the high molecular weight subunits (HMW-GS) and the low molecular weight subunits (LMW-GS), with MWs range of 75 to 120 kDa and 30 to 74 kDa, respectively (Fig. 1.6) [19, 24, 32, 33]. The LMW-GS comprise about 20 to 30% of the total gluten proteins, instead the HMW-GS are minor components, accounting for about 5-10% of the total proteins. Despite their abundance, the LMW-GS have received much less research attention than the HMW-GS. This has been mainly due to the difficulty in identifying them in one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The problem, which was principally due to overlapping between LMW-GS and gliadins, was largely resolved using a two-dimensional electrophoresis procedure, RP-HPLC techniques, capillary electrophoresis and also mass spectrometry [13, 46]. Contrarily, HMW-GS have been studied in most detail because they have the greatest impact on dough elasticity, which is the major determinant of breadmaking performance [4, 26, 35, 37].

The amino acid compositions of glutenins are very similar to those of gliadins, with high levels of glutamine and proline and low levels of charged amino acids. The sequences of purified glutenins reveal that they all have at least two or three distinct structural domains: a central repetitive domain, flanked by non-repetitive C-terminal and N-terminal domains. Based on their amino acid sequences, the LMW-GS resemble α/β- and γ-gliadins. The N-terminal domain consists of glutamine- and proline- rich repetitive sequences (QQQPPFS), while the C-terminal domain is homologous to those of α/β- and γ-gliadins, respectively [19, 25, 30]. Besides, LMW-GS contain eight cysteine residues, which are considered to be involved in the formation of both intra- and inter-chain disulfide bonds. For steric reasons, some cysteine residues are not able to form intra-chain disulfide bonds and, consequently, inter-chain disulfide bonds between different gluten proteins are generated. It was found that only two cysteine residues, one located at the N-terminal end and the second one located near the C-terminal end, form inter-chain disulfide bonds [21, 30, 39]. The LMW-GS have also been

further classified into three subgroups (B, C and D) based on gel electrophoretic mobility on SDS-PAGE under reduced conditions and their pI. The subgroups B and C have MWs of 42–51 kDa and 30–40 kDa, respectively. Their amino acid sequences are closely related to the amino acid sequences of α- and γ- gliadins. Highly acidic LMW-GS, having the MWs of 52-74 kDa, are present in the subgroup D and are related to ω-gliadins in term of electrophoretic mobility and N-terminal sequence (Fig. 1.6) [13, 19, 25]. The LMW-GS are encoded by genes at the Glu-A3, Glu-B3 and Glu-D3 loci on the short arm of chromosomes 1A, 1B and 1D, respectively. Chromosome 1A encodes relatively few LMW-GS, instead there is extensive polymorphism for LMW-GS encoded by chromosome 1B. There is evidence that LMW-GS synthesis is also controlled by genes on the group 6 chromosomes. The LMW-GS genes, like all other prolamin genes, do not have introns in their sequences [54]. Also the secondary structures of LMW-GS, except for D-subunits, have an overall similarity to the structure of S-rich gliadins. N-terminal repetitive domains are rich in β-turns, possibly forming a regular spiral structure, while the short non repetitive domains are rich in α-helix and appear to be more compact. Based on their structure, the LMW-GS have the ability to form large aggregates that are related to dough strength [13, 30].

HMW-GS are minor components within the gluten proteins family in terms of quantity, but are major determinants of gluten elasticity [19, 34]. It has been found that dough properties are strongly influenced by the quantity and the type of HMW-GS. They are the largest polypeptides, very difficult to solubilize, and contain the highest level of glycine residues among gluten proteins [13, 25]. Each common wheat cultivar possesses three to five HMW-GS, also known as A subunits, which can be grouped into two different types: the x- and the y- type [10, 23, 30]. These are encoded at the Glu-1 loci on the long arms of the group 1 chromosomes (1A, 1B, and 1D). Each locus includes two genes linked together encoding for both the x- and the y-type subunits. The x-type subunits generally have slower electrophoretic mobility and higher MWs (83-88 kDa) than the y-type (67-74kDa) subunits [19, 30, 52]. Both HMW-GS and LMW-GS genes exhibit extensive allelic variations which impart different effects on gluten quality [13, 19, 23, 34]. HMW-GS have high contents of proline and glycine, and low contents of lysine with the unusually high content of glutamic acid. Structurally they consist of non repetitive N- and C-terminal domains flanking a central repetitive domain (480-700 residues). The central domain contains repetitive hexapeptides (PGQGQQ) as a backbone with inserted nonapeptides (GYYPTSPQQ) and tripeptides (GQQ). The hexapeptides often occur in tandem arrays, whereas the nonapeptides and tripeptides always occur in an interspersed way. The hexapeptides and nonapeptides are present in both x- and y-type subunits, while the tripeptide motif is only present in the x-type [23, 26, 19]. The N- and C-domain are characterized by the frequent occurence of charged residues and contain most, or all, of the cysteine residues present in the subunits

[13, 30]. In particular, the x- type subunits contain three cysteine residues in the N-terminal domains and one in the C-terminal domain. Instead, the y-type has five cysteines in N-terminal domain and one in each of repetitive and C-terminal domain [26, 34]. Furthermore, several x- and y-type subunits contain an additional cysteine residue in the central repetitive domain. These cysteine residues are involved in the formation of both intra- and inter-chain disulfide bonds. It has also been postulated that the intra-chain bonds form more rapidly than inter-chain links [19, 25, 30].

Whit regard to the secondary structure of HMW-GS, various approaches have been applied. In particular, studies of the repetitive domain indicate the presence of β-reverse turns. Instead, N- and C-terminal regions were proposed to have a globular structure with α-helices [19, 21, 26, 30]. Furthermore, the subunits of glutenin are cross-linked in a head to tail fashion to form a linear macropolymers of glutenin known as 'concatenations'. The crosslink found between the HMW- and LMW-GS is a disulfide bond between a cysteine residue in the y-HMW-GS repetitive domain and a cysteine residue in the C-terminal region of LMW-GS [25, 30, 39]. Differences in glutenin subunits size, polarity, and number of cysteine residues influence the ability to form disulfide bonds necessary for building up the glutenin polymer structure. This variation is a critical factor in determining bread dough end-product quality. Glutenins apparently impart to dough its property of resistance to extension. The HMW-GS make doughs elastic and allow them to trap the gas bubbles produced by yeast and to rise [4, 19, 34]. Besides, among the HMW-GS the contribution to x-type to the dough proprieties has been found to be more important than that of y-type [10, 30].

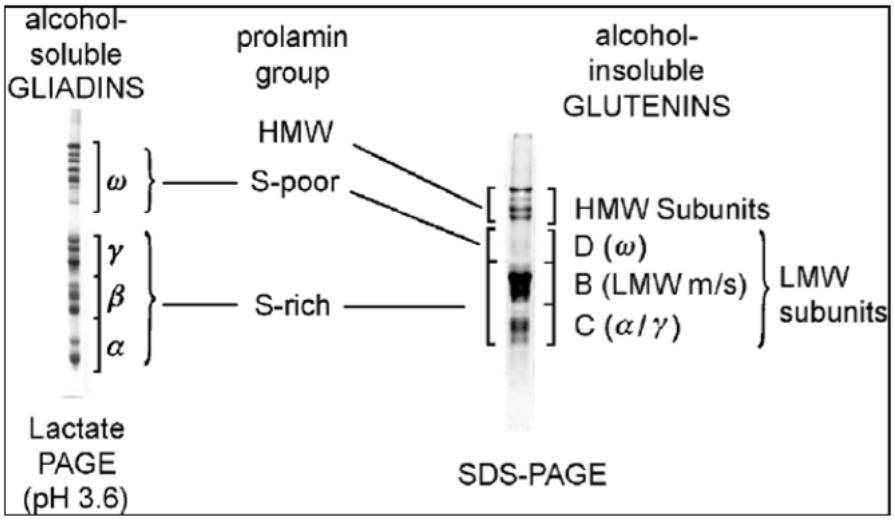


Fig. 1.6. Classification of gluten proteins: monomeric gliadins and polymeric glutenins. ω, γ, β and α indicate subunits of gliadins; S- indicates cysteine residues; B-, C-, D-LMW indicate low molecular weight subunits of glutenins; HMW indicates high molecular weight of glutenins (modified from [19]).

The baking process produces precise changes in the glutenin structure and functionality, which are still not completely understood. Oxygen is known to be essential for the formation of large glutenins polymers. In molecular terms, these changes may include some disulfide interchange reactions during dough mixing, resulting in different contents of the sulphydryl (-SH) and disulfide (S-S) group [10, 19, 34].

2. Wheat flour: nutritional qualities and health effects

Wheat is the most important staple crop in temperate zones, and the number of people who eat wheat for a substantial part of their diet reaches several billion worldwide. Increasing demand for wheat is based on the ability to make unique food products whose growing consumption is also due to industrialization and westernization. In particular, the unique properties of the gluten protein fraction allows the processing of wheat to produce bread, other baked goods, noodles, pasta, and a range of functional ingredients. Furthermore, wheat is the main source of energy for all age groups, with bread alone contributing about a fifth of the average daily intake [20, 25, 38]. Wheat provides 30–35% of energy intake in the UK and up to 60% of the daily calories in developing countries [58].

Wheat grains provide a range of components with an established essential or beneficial role for human health. It is also a fermentable substrate for the human colonic microflora, which conveys substantial benefits to the host [31]. Wheat nutrient contents and compositions are strongly influenced by genotype, environment and genotype-environmental interactions [3]. Because of the high content of starch, (about 60%–70% of the whole grain and 65%–75% of white flour) wheat is often considered no more than a source of calories. However, despite its relatively low protein content (usually 8%–15% of the dry weight), wheat still provides as much protein for human and livestock nutrition as the total soybean crop [20, 33, 36, 55]. The protein content of wheat varies widely. Although a third of this variation is influenced by genetics, the major impact is related to the availability of nitrogen during the wheat growing [33, 36]. Protein nutritional quality is determined by the proportions of essential amino acids, as these cannot be synthesized by animals and hence must be provided in the diet. Nine amino acids are strictly essential: lysine, isoleucine, leucine, phenylalanine, threonine, tryptophan, valine, histidine, and methionine [20]. Comparison with the values for whole wheat grain and flour shows that only lysine and tryptophan are deficient, with some essential amino acids being present in considerably higher amounts than the requirements [4, 16, 38].

Wheat also contains vitamins (B vitamins), dietary fiber and minor components including lipids, phytochemicals and minerals which may contribute to a healthy diet. Most of them are concentrated, or solely located, in the bran [20, 38]. The B vitamin complex comprises eight water-soluble components which often occur together in the same foods. Cereals are dietary sources of several B

vitamins, particularly thiamine (B1), riboflavin (B2), niacin (B3), pyridoxine (B6) and folates (B9). All B vitamins are concentrated in the wheat bran and/or germ, with white flour containing significantly lower contents than wholemeal [20]. Dietary fibers derive from polymers of the wheat endosperm cell wall and are constituted mainly by arabinoxylans (roughly 69%) and (1-3) (1-4) β-D-glucans. The arabinoxylans, present in both soluble and insoluble forms, show antitumor activity and immunostimulatory effect in cancer prevention. In general, dietary wheat bran may have a protective effect against colon tumor development because it is able to increase the fecal bulk, thereby diluting potential carcinogens and tumor promoters in the colon lumen [38, 57]. Lipids are present only in a small extent in wheat but they have a significant effect on the quality and the texture of foods because of their ability to associate with proteins and starch [16]. Furthermore, wheat bran is a rich source of phytochemicals and lipid-soluble components with healthy proprieties. Among these, phenolic acids represent, quantitatively, the major group of phytochemicals in the wheat grain, ranging up to almost 1200 μg g^{-1}. Epidemiological studies indicate that they have an important antioxidant activity. Lignans, present at levels up to 10 μg g^{-1} in wholemeal wheat and almost 20 μg g^{-1} in the bran, are polyphenols with phytoestrogen-like activity instead, folates positively correlate with the bran yield [38]. Finally, wheat is a source of minerals such as zinc (20–30 mg Kg^{-1}), iron (30–36 mg Kg^{-1}) and selenium (from about 10 μg Kg^{-1} to over 2000 μg Kg^{-1}) (FAO/WHO, 2001; [56]). The concentration of selenium in wheat is largely determined by the availability of this element in the soil. Consequently, wheat produced in Western Europe may contain only one-tenth of the selenium that is present in wheat grown in North America [4]. Generally, conventional processing of the grains removes most of the micronutrients. Furthermore, over three billion people are currently micronutrient and vitamins malnourished. This global crisis in nutritional health is the result of dysfunctional food systems that do not consistently supply enough of these essential nutrients to meet the nutritional requirements of high-risk groups. For this reason, many countries have mandatory fortification for iron and selected vitamins in flours from wheat, maize and rice. Fortification of cereal flour with zinc is also practiced in several countries, but largely on a voluntary basis [16, 58].

Despite wheat nutritional qualities, a healthy diet obviously requires supplementation with other food groups such as legumes, vegetables, fruits, or animal products to make the diet more balanced and adequate, particularly concerning vitamin A, iron, and riboflavin (B2) [20]. Even though its important nutritional impact, wheat is listed among the ‘big eight’ food allergens which together account for about 90% of all allergic responses IgE-mediated. Although some wheat gliadin proteins have been reported to be responsible for allergic reactions to the ingestion of wheat products, the incidence of true food allergy is fairly infrequent in adults, but it affects up to 1% of children. On the other hand,

wheat proteins are certainly known as the most significant environmental factors that trigger celiac disease (CD) in genetically-predisposed individuals [4, 19].

3. Celiac Disease

3.1. An overview of Celiac Disease

Celiac disease (CD) is a chronic small intestinal immune-mediated enteropathy triggered by the ingestion of dietary gluten in genetically predisposed individuals. Gluten is the protein component present in various cereals, including wheat, rye, barley, spelt, and kamut. The ingestion of gluten induces an inflammatory process that results in the damage of the gut mucosa. This condition involves villous atrophy and crypt hyperplasia in addition to the infiltration of a consistent number of lymphocytes and plasma cells in the *lamina propria* [61-68, 77]. CD is also known under several names: celiac sprue, non-tropical sprue, endemic sprue, gluten enteropathy or gluten-sensitive enteropathy, gluten intolerance and idiopathic steatorrhea [70, 74, 77]. The name "sprue" was firstly used in the 18th century and is derived from Dutch word spruw, meaning aphthous disease, due to the high prevalence of aphthous mouth ulcers in celiac patients. Francis Adams introduced the term “coeliac”, recently more often spelled as celiac. Although symptoms and signs of CD have been recognized for more than 100 years, it was in the 1940s that the Dutch paediatrician Dicke established a link between gluten exposure and CD. The first consensus definition of CD was published in *Acta Paediatrica* in 1970. This publication defined CD as a permanent condition of gluten intolerance with mucosal flattening that reversed on a gluten-free diet and then relapsed on re-introduction of gluten. CD and disorders related to gluten ingestion are now common chronic diseases in children and adults, and increased diagnosis has led to a proliferation of research activities. As with many other chronic conditions, the boundaries of CD are not always distinct and its pathogenesis remains unclear [61, 67, 73, 87].

Signs and symptoms of CD result in a variable degree of intestinal and extra-intestinal damages, which complicate the diagnosis of CD. Symptoms vary, with some patients having diarrhea and malabsorption, others suffering from constipation, fatigue, and depression and some are asymptomatic. Generally, the intestinal mucosal injuries and nutrient malabsorption have widespread consequences, linked to diverse manifestations that include osteoporosis, lymphoma, pneumonia and increased mortality [61, 62, 64]. The only currently available treatment of CD is a lifelong strict gluten-free diet, which is difficult to maintain and can lead to social isolation because modern diets are heavily based on products that contain gluten [68, 77, 82].

3.2 Epidemiology

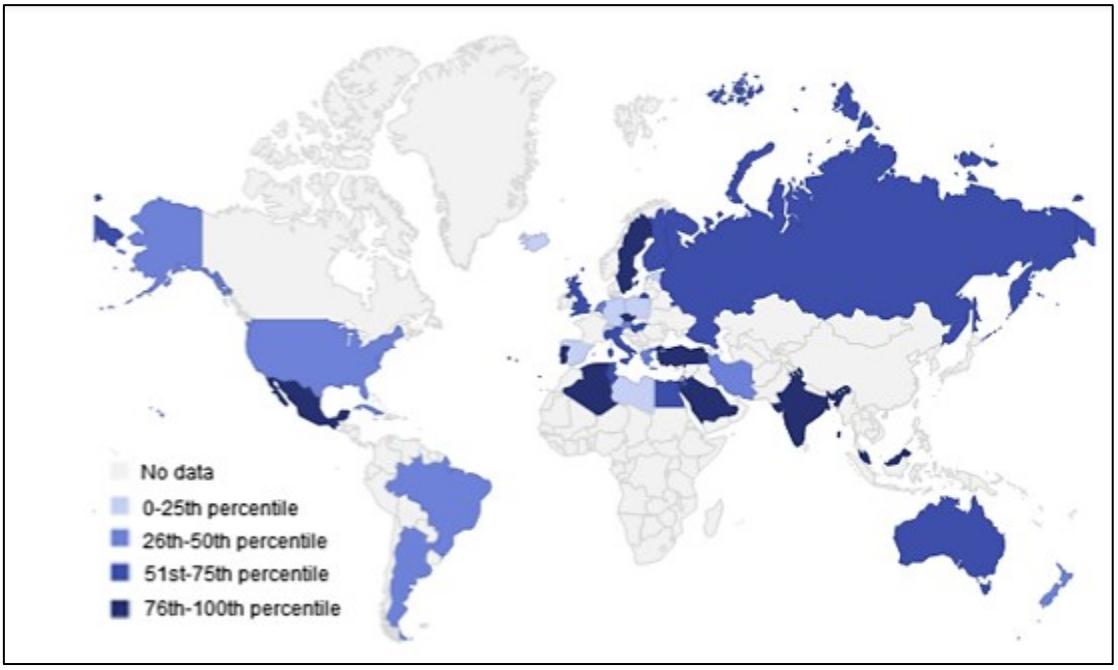
Historically, CD was considered a rare condition, with a prevalence of about 0.02%, characterized predominately by intestinal symptoms that led to a malabsorption syndrome and stunted growth in young children of European ancestry. However, it is now appreciated that CD has a worldwide and increasing incidence among persons of various ethnic groups and among both children and adults, except for areas showing low frequency of CD-predisposing genes and low gluten consumption. Nowadays, the global prevalence of CD based on serologic test results is about 1–1.4% and based on biopsy results is 0.7% (Fig. 1.7) [62, 81-83, 88]. The exact percentages of CD seroprevalence vary by country: 0.3% in Germany, 0.7% in Italy, 1.2% in England, and 2.4% in Finland. Besides, the prevalence is higher in first-degree CD relatives (10–15%) and other at-risk groups (5-10%), particularly in patients with Down and Turner syndrome, type 1 diabetes, autoimmune thyroid disease. The reason for the recent increases in the number of reported cases may be in part due to changes in diagnostic practice. It seems to be increasing from fourfold to fivefold over the past 50 years [64, 71, 72, 86]. Nevertheless, about 85% CD cases still remain undetected in the absence of serological screening due to heterogeneous symptoms and/or poor disease awareness [71, 82]. Although often thought to be European in origin, the distribution of CD also extends beyond European borders, with significant prevalence identified in such disparate populations as the Middle East, Asia, South America, and North Africa. The prevalence values of biopsy-confirmed CD are 0.4% in South America, 0.5% in Africa, 0.6% in Asia and 0.8% in Oceania [88]. A large multicentre study in the United States found a prevalence of 0.75% in not-at-risk groups, rising to 1.8% in symptomatic people, 2.6% in second-degree relatives of a person with CD and 4.5% in first-degree relatives. This profile is similar to the prevalence in Europe. The rate amongst adult blood donors in Iran, Israel, Syria, and Turkey is 0.60%, 0.64%, 1.61%, and 1.15%, respectively. People of African, Japanese and Chinese descent are rarely diagnosed. This reflects a much lower prevalence of the genetic risk factors. Instead, people of Indian ancestry seem to have a similar risk to those of Western Caucasian ancestry [63, 71, 73]. One proposed reason for this trend is that, with a globalizing world market, developing nations that traditionally relied on rice and maize are increasingly incorporating wheat-based foods into their diets. It is now that the medical community across Asia should define the extent of the problem and prepare to handle the impending epidemic of CD in Asia [84, 85]. As more mass screening studies are performed in different populations, more cases of previously undiagnosed CD are identified, even though the percentage of people with clinically diagnosed disease represents the tip of the iceberg [83-89].

CD is a disorder affects people of any age or gender, but sex differences also exist concerning the rate of diagnosis with a female/male ratio of 2 to 3:1. The differential rates of diagnosis among sexes

is thought to reflect several factors, including a higher rate of autoimmune disease among women in general, more regular health care interaction in female than male subjects, and a higher likelihood of symptomatic disease among women than men [72, 86-88].

Among the pediatric population, diarrhea and malabsorption syndrome are seen in the very young, whereas growth issues occur in children of all ages. Recurrent abdominal pain and other clinical presentations are more common in older children. Interestingly, the prevalence of CD among Finnish children is at least 1 case in 99 children. One explanation for this high prevalence might be that the Finnish population studied may have had an unusually high genetic risk of CD. The age at diagnosis has also increased over time in the pediatric population [71, 89]. Although the absolute number of patients with CD at present is not very high, this number is expected to increase markedly over the next few decades owing to increasing awareness, advances in population screening and diagnosis techniques.

A



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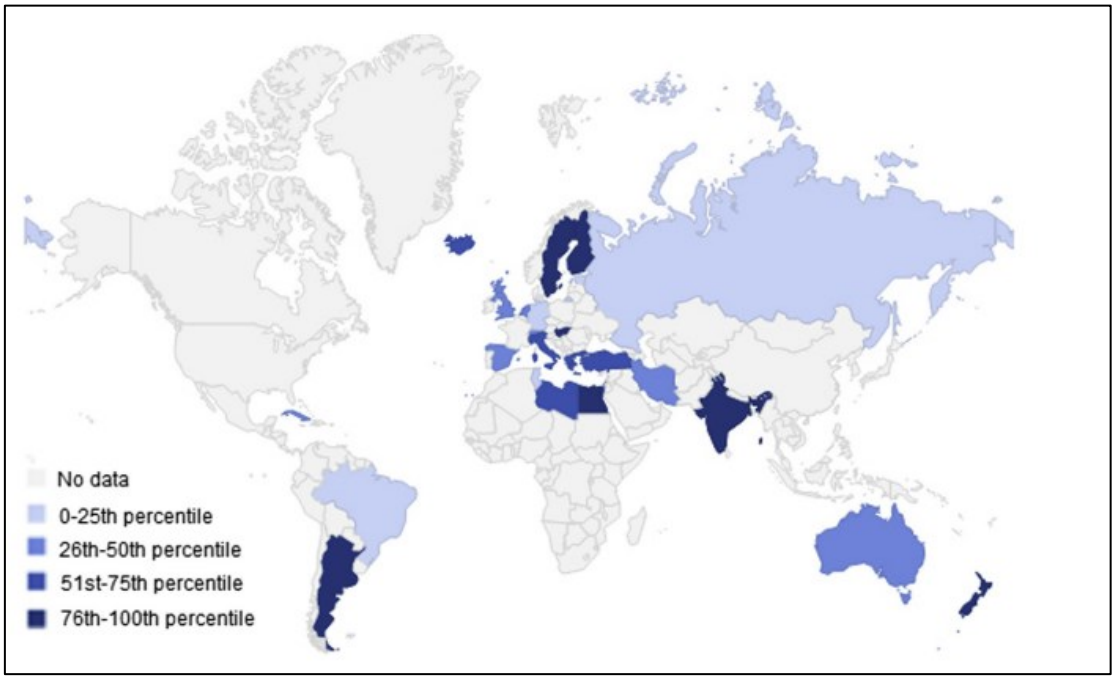

Fig. 1.7. A. Worldwide celiac disease seroprevalence rates for the countries reporting data. **B**.Worldwide celiac disease prevalence rates, based on biopsy, for the countries reporting data. Prevalence values were stratified into 4 groups of percentiles [88].

3.3 Etiopathogenesis

CD is a small intestinal autoimmune enteropathy triggered by the interaction of genetic and environmental factors. In genetically-susceptible individuals, the ingestion of dietary gluten induces an inflammatory process, with specific serological and histological profiles, that results in the final damage of the bowel mucosa. The main histopathological features are villous atrophy and crypt hyperplasia in addition to infiltration of a consistent number of lymphocytes and plasma cells in the *lamina propria* respectively [77, 98]. The inflammatory process induces remodeling in the structure and function of the small intestinal mucosa. In particular, the main structural changes are characterized by the thinning of the gut mucus layer [65, 67], the morphological alteration of tight junctions (TJs), which seal together the epithelial cells, and the apoptosis of enterocytes up to completely villous atrophy. Furthermore, in the CD mucosa, there is an inversion in the normal differentiation and proliferation program of the tissue. Biopsies highlight a reduction in the differentiated compartment up to complete villi atrophy and the increase in the proliferative compartment along with crypt hyperplasia (Fig.1.8) [63-67]. Instead, functional changes involve a quali-quantitaive modification of intestinal microbiota composition, an alteration of gut barrier function leading to an increase of the intestinal permeability, a condition known as leaky gut, malabsorption of nutrients and the dysregulation of intestinal homeostasis [127, 128].

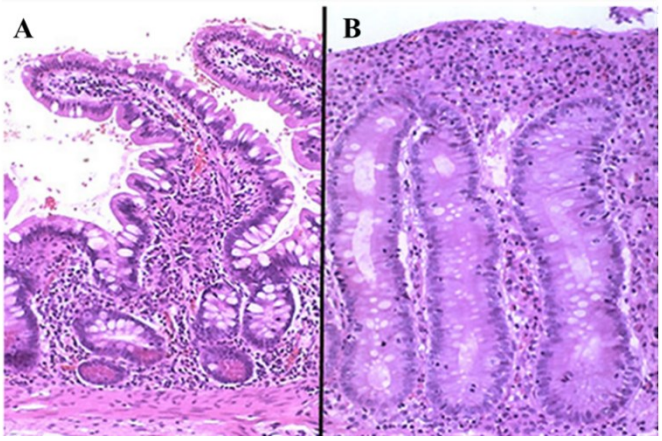

Fig. 1.8. Upper small intestine biopsy. A: normal small intestine. B: villous atrophy of CD patient (modified from [71]).

The etiology of CD is multifactorial with pathogenesis still not well defined [109, 114]. The first factor is the genetic susceptibility to disease. CD is associated with specific Major Histocompatibility Complex (MHC) class II alleles, on chromosome 6p21, that encode for Human Leukocyte Antigen (HLA) [96, 98]. More than 90% of CD patients carry a combination of HLA-DQA1*05:01and HLA-DQB1*02 alleles, in either the *cis* or the *trans* configuration, and the gene products together form the HLA-DQ2 heterodimer. HLA-DQ2 homozygosis confers a much higher risk (25–30%) of developing early-onset CD in infants with a first-degree family member affected by the disease [82]. Most of the HLA-DQ2 negative CD patients carry the HLA-DQA1*03 and HLA-DQB1*03:02 alleles, encoding the HLA-DQ8 heterodimers. HLA-DQ8 characterizes the remaining 5–10% of celiac individuals [72, 77, 91]. Despite HLA-DQ2 or HLA-DQ8 are expressed in 30–35% of the general population, only 2–5% of gene carriers developing CD. This implicates other genetic and epigenetic as well as environmental factors as contributors to the manifestation of the disease [68, 90]. Several data underline the importance of non-HLA genes in CD risk. More than 70 candidate genes in 42 non-HLA loci have been implicated in CD heritability based on genome-wide association studies and follow-up studies. The individual contribution from each of these genes is small and collectively they account for roughly 15% of the additional disease risk. Despite the small contribution, these loci highlight the importance of several immune pathways in CD pathogenesis, including roles for T- and B-cell activation, chemokine receptor activity, and cell migration, cytokine binding, thymic differentiation of CD4+ and CD8+ T cells, stress pathways and innate immunity [72, 91, 96]. For this reasons CD is also classified as a polygenic disorder.

Nevertheless, HLA predisposing genes are necessary for disease development but not sufficient. In fact, the environment clearly plays a crucial role in the development of the CD. The gliadin fraction of wheat gluten represents the well studied environmental factor responsible for the development of

the intestinal damage, typical of the CD [63- 67, 91]. There are at least 50 toxic epitopes in gluten peptides exerting cytotoxic, immunomodulatory, and gut-permeating activities. These activities have been partially mapped to specific domains in α-gliadin. The cytotoxic peptide 31–55 (25-mer), the immunomodulatory peptide 57–89 (33-mer), the chemokine receptor (CXCR3) -binding zonulin-releasing peptides 111–130 and 151–170, and the interleukin (IL)-8-releasing peptide 261–277 [66, 110, 130]. Only two main peptides remain undigested: the 33-mer (P55–87) and the 25-mer (P31–55). Consequently, these two peptides are the main peptides that are active *in vivo* in the celiac intestine after gluten ingestion [66, 80]. The 33-mer α-gliadin fragment (sequence LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) is the most immunogenic peptide because it harbors six overlapping copies of three different HLA-DQ2 restricted T-cell epitopes. It is also resistant to the enzymatic degradation by gastric acidity and pancreatic and brush-border peptidases [96,113]. When a condition of altered gut permeability occurs in genetically susceptible individuals, this peptide might reach the immune districts in the *lamina propria* of intestinal mucosa in an intact and stimulatory form and severe intestinal damage ensues [80, 129]. Under physiological circumstances, the intestinal epithelial barrier is almost impermeable to macromolecules and this interplay is prevented by competent intercellular TJs. However, the CD is characterized by enhanced paracellular permeability across the intestinal epithelium, leaky gut condition, that would allow passage of macromolecules through the paracellular spaces [65]. After food ingestion, undigested gliadin peptides cross the epithelium and reach the *lamina propria* where they were deaminated locally by the calcium-dependent enzyme Tissue Transglutaminase 2 (TG2). Like other transglutaminases, the TG2 cross-link proteins via the formation of covalent bonds between lysine and glutamine amino acids [98]. In addition, the TG2 promotes in the gluten peptides the selective conversion of specific glutamine residues into glutamic acid. Thus, in genetically predisposed subjects, the presence of acidic residues with negative charges at characteristic positions allow to bind efficiently HLA-DQ2 or HLA-DQ8 molecules on the surface of Antigen Presenting Cells (APCs) [65, 77, 91-93]. The molecular basis for the linking of CD with HLA-DQ is thought to be related to the physicochemical properties of MHC molecules. They have positively charged pockets that can bind strongly to the negatively charged molecules [130]. The mature APCs are able to directly present, without needing intracellular processing, the immunogenic deaminated peptides to CD4+ T-helper lymphocytes (CD4+ T) in the *lamina propria* [113]. CD4+ T cells efficiently recognize the immunogenic epitope by their T cell receptor (TCR), a heterodimer consists of one α− and one β−chain, then activate and differentiate into CD4+ T-helper 1 (Th1) lymphocytes. This results in the adaptive immune response activation with subsequent release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interferon-γ (IFN-γ), IL-21, IL-22 by Th1

and matrix metalloproteinases (MMP) by the myofibroblasts. This leads to tissue damage with further increases the epithelial permeability and lymphocytic infiltration into the submucosa [76, 95]. Normally, gluten peptides cause a Th2 immune response whereas in CD patients induce a Th1 cell response in the presence of deamidated gluten peptides in APCs [111-114, 130]. Moreover, Th1 cells are able to activate B cells which differentiate in plasma cells and release IgA antibodies against TG2 (tTG), gliadin (AGA) and endomysium (EMA). These antibodies are typically present in the sera of most patients and can be used for diagnostic purposes. The finding that TG2 not only selectively modifies gluten peptides but is also a direct target of autoimmune antibodies is intriguing [91, 102].
After activation, T CD4+ cells stimulate, by high levels of pro-inflammatory cytokines, natural killer (NK) cells and CD8+ intraepithelial T lymphocytes (IELs) which, in turn, promote the typical mucosa remodeling and damage. The damage to the intestinal mucosa, in turn, activates more of TG2 that will alter more of gliadin peptides into negatively charged particles, promoting inflammatory loop and amplifying ongoing damage [77, 130]. Although the role of HLA class II-restricted CD4+ T cells in CD pathogenesis is well documented, evidence also supports the involvement of HLA class I-restricted CD8+ T cells [67].
Several studies demonstrate that the inflammation of the intestinal mucosa is due not only to the derangement of adaptive but also to the innate immune responses to wheat gliadin. The peptide P31-43 contained in the 25-mer is not recognized by the T cells, but, it induces stress and an innate immune response with production of IL-15 by the macrophages, dendritic cells and enterocytes as primary mediator. IL-15 is one of the key regulators of the immune response in CD. IL-15 plays a role in the loss of oral tolerance to gluten, inhibiting the activity of CD4+ CD25+ FoxP3+ regulatory T cells (Treg) in the intestinal mucosa of CD patients and promoting the development of Th17 cells, NK and IELs [114, 118, 130 -134]. IELs represent a heterogeneous population of T cells composed mainly of cytotoxic CD8+T cells residing within the epithelial layer, whose main role is to maintain the integrity of the epithelium by eliminating infected cells and promoting epithelial repair. Dysregulated activation of IELs by innate and adaptative signals is critically involved in epithelial cell destruction and the subsequent development of villous atrophy [133]. In addition to IL-15, also IL-8, released by monocytes and activated dendritic cells CD103+ (DCs), plays an important role in the immune response of CD patients, acting as a chemotactic factor for T lymphocytes [64, 139]. Instead, IFN-γ released by Th1 cells is linked to mucosal damage in CD patients [130, 131].
Furthermore, the P31-43 can induce proliferation of enterocytes via epidermal growth factor (EGF), resulting in crypt hyperplasia, one of the characteristic findings in celiac mucosa remodeling [66, 130]. Generally, gliadin peptides induce alterations of structure, signaling and proliferation and stress/innate immunity activation in several intestinal cell lines [63-67]. The gliadin peptides can also

directly stimulate the innate immune response of macrophages and dendritic cells through pattern recognition receptors (PRR), such as toll-like receptors (TLRs) 4. This activation leads to the maturation of these cells and to the secretion of inflammatory cytokines (e.g., IL-1β, IL-8, TNF- α and MCP-1) which enhance the adaptive immune response against gluten (Fig. 1.9) [92-95].

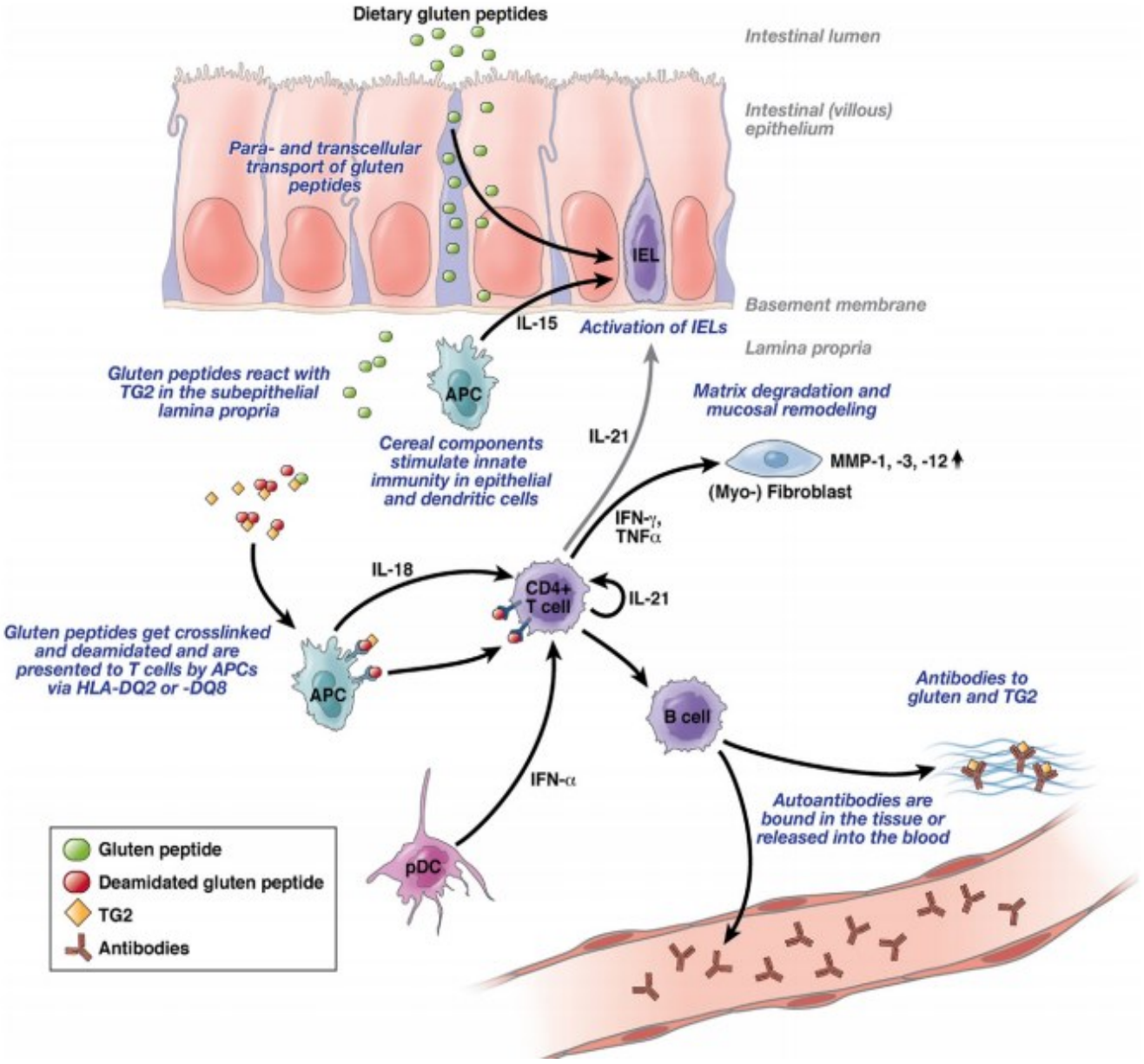

Fig. 1.9. **Schematic representation of the pathogenic mechanism involved in the onset of celiac disease**. The development of the autoimmune response, which causes celiac disease in genetically susceptible individuals, needs the passage of gliadin peptides through the intestinal barrier either paracellularly or intracellularly. In the submucosa, peptides are deamidated by tissue transglutaminase and then processed by antigen-presenting cells and exposed on their surface within the HLA-DQ2 or HLA-DQ8 heterodimer. This presentation activates CD4+ T cells that, in turn, trigger the adaptive immune response with effector T-cell activation and autoantibody production. The passage of the gliadin peptides will also induce the activation of innate immunity, mainly mediated through IL-15 release [68].

Common denominator of CD is the presence of a variety of pre-existing conditions leading to an autoimmune process. The first component needed to develop an autoimmune process is a genetic susceptibility for the host immune system. Secondly, the host must be exposed to the antigen. Finally, the antigen must be presented to the gastrointestinal mucosal immune system following its paracellular passage (normally prevented by the TJs competency) from the intestinal lumen to the gut submucosa. In all cases, increased permeability is also a key factor in the onset of CD. A leaky gut allows the entry of exterior antigens from the gut lumen into the host, which may promote both local and systemic immune responses [63, 65]. Numerous factors can affect gut permeability, such as various diet-derived compounds, infections, infant feeding, alcohol consumption, and gut dysbiosis [127]. In particular, certain infections (such as Rotavirus among infants and Campylobacter among adults) have recently been shown to be associated with an increased risk of CD. Normally, bacterial or viral products on the intestinal surface are usually detected by specific TLRs, leading to the activation of various intracellular cascades and thus the induction of immune response. The prolonged inflammatory state owing to infections could induce tissue damage, thus the alteration of the intestinal barrier and increased permeability among epithelial cells. This allows the entry of gliadin across the lumen to reach the *lamina propria* [73,130, 135]. Interestingly, increased expression of TLR4 and TLR2 was present in CD gut, suggesting a role of infections and dysbiosis in the initiation of intestinal barrier damage [92, 130, 132, 135]. Moreover, children who were born via elective caesarian section are at increased risk of developing CD, while those born via emergent caesarian section (and may have had contact with the birth canal) are not. It is hypothesized that caesarian section has prolonged consequences for the quali-quantitative alteration in the composition of the intestinal microbiota leading to a dysregulation of the immune system in the first year of life. However, the conclusions on this subject are still contradictory [121-123, 130]. Also antibiotics, proton pump inhibitors, and surgical operations are each associated with an increased risk of development of CD [137]. They could disrupt the integrity of the gut barrier and alter microbiota which is necessary for the development of an appropriate immune response [124, 127, 130, 135].

Several studies underline the presence of a balance existing between commensal bacteria and the mucus layers, which together contribute to the maintenance of gut homeostasis and oral tolerance against food antigens [100, 127, 131, 132]. Microbial colonization occurs at birth and shapes the development of the mucosal and systemic immune system and the intestinal barrier. These host-microbe interactions continue throughout life, and disruption of these sensitive balances, through altered bacterial composition or functions, have been hypothesized to increase the risk of a range of autoimmune and inflammatory diseases such as CD [131, 132]. Furthermore, also the epithelial and mucus layers are in intimate relation with the innate and adaptive immune system and are directly

involved in the induction of oral tolerance. There is not only communication from the epithelial cells to the immune system but also in the opposite direction. Mucus and intestinal epithelium are the primary gatekeepers and controllers of bacterial interactions with the host immune system. [100, 101, 136]. The major component of the outer intestinal mucus layer is the mucin 2 (MUC2), a gel-forming glycoprotein secreted by goblet cells, which not only constitutes a physical barrier against infections, but also serves as nutrient and a binding site for commensal bacteria, and enhances gut homeostasis [79, 100, 138].

In the final analysis, the pathogenesis of CD involves complex dysregulated interactions between a range of factors. Genetic predisposition and exposure to gluten, loss of intestinal barrier function, inappropriate adaptive and innate immune response, and an imbalanced gut microbiome all seem to be key elements for the onset of CD [78, 80, 82, 113]. Removal of gluten from the diet is an effective way to stop the disease process. Nowadays, the only available treatment consists of lifelong adherence to a gluten-free diet [77, 91].

3.3.1. Cytokines and their role in celiac disease

The immune system is a complex entity that is constantly integrating signals from a multitude of sources. The cytokines represent the language understood by immune cells. In the healthy human gut, there is a steady state of immune homeostasis, which is missing in CD. Indeed, in CD the immune system initiates a process that leads to the loss of oral tolerance to gluten and licensing of all immune cells to mount an inflammatory response [74]. This process is also dictated by cytokines released by innate immune cells and by $CD4^+$ Th1 or Th2 lymphocytes subpopulation. In particular, IL-15 and interferon-alpha (IFN-α) are two cytokines, produced by the cells of innate immunity, that have the potential to influence multiples levels of the immune system by acting on APCs, intestinal epithelial cells and IELs [165]. In particular, IL-15, a pro-inflammatory cytokine, has the ability to polarize DCs to the development of inflammatory response [113, 115, 130]. It is involved in the expansion and survival of TCR αβ IELs promoting the expression of anti-apoptotic molecules [74, 163, 164]. Studies show that IL-15 is upregulated in active CD in both the *lamina propria* and epithelium. IL-15 with IFN-α contribute to intestinal dysbiosis and loss of tolerance, which in turn influence the release of cytokines [116, 117, 165]. IL-12, IFN-α, IL-6, tumor necrosis factor-alpha (TNF-α) and other proinflammatory cytokines released by macrophages act on $CD4^+$ Th1 cells promoting the secretion of interferon-gamma (IFN-γ), a type II interferon, which in turn is an important activator of macrophages. $CD4^+$ Th1 cells are also able to release TNF-α and IL-6, in addition to other proinflammatory cytokines such as IL-1β, IL-21, IL-22 that activate cytotoxic $CD8^+$ T cells responsible for tissue remodeling and damage [130]. IL-2, released by $CD4^+$T cells, acts in a paracrine

way stimulating their differentiation in Th1 subpopulation. Instead, $CD4^+$Th2 associated cytokines are IL-4 and IL-5. IL-4 acts with a positive feedback loop inducing the differentiation of $CD4^+$ T helper naive to $CD4^+$Th2. It is a key regulator in humoral and adaptive immunity and with IL-5 induces B cell differentiation and isotype switching [170]. In the mucosa of patients with active CD, mRNA coding for IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-18, IL-21, IL-22 are overexpressed [25, 167-170]. In addition to proinflammatory mediators, during an immune response $CD4^+Foxp3^+$Treg release immunoregulatory cytokines, such as transforming growth factor-beta (TGF-β) and IL-10, that suppress the inflammatory process and contribute to immune homeostasis. In particular, TGF-β reduces T cell proliferation and differentiation. Impaired TGF-β signaling is associated with the development of inflammatory bowel disease (IBD) [166]. IL-10 is a pleiotropic cytokine with potent anti-inflammatory properties which induces the development of DCs with a tolerogenic phenotype that in turn limit T cell activation or promote T cell anergy. Moreover, IL-10 prevents IELs infiltration and epithelial damage in the small intestine of celiac patients. Alterations in IL-10 secretion or in its receptor (IL-10R) lead to the loss of oral tolerance [168].

3.3.2. The Intestinal Barrier

The intestinal barrier (Fig. 1.10) separates the lumen from the underlying tissues and is formed by a monolayer of epithelial cells, including enterocytes, Paneth, endocrine, goblet, and M cells [76-78] All these specialized cells are sealed together by the junctional protein complexes to form a continuous and polarized cell monolayer. The junctional complexes that contribute to the integrity of the intestinal barrier, are mainly formed by desmosome, adherents (AJs) and tight junctions (TJs). The AJs and desmosome provide essential adhesive and mechanical properties that contribute to barrier function but do not seal the paracellular space. Instead, the TJs, located most apically on the lateral membrane, are required for the proper formation of epithelial cell polarity as well as sustaining the mucosal barrier. Furthermore, TJs are the key cell junctions modulating the rate-limiting step of paracellular transport [79, 108, 140].

The gut barrier acts as a selective semi-permeable barrier and it is able to discriminate between solutes based on size and charge [141]. It regulates the absorption flow of nutrients, ions, and water between the lumen and underlying tissues and plays an important role in the first line of host defense against a variety of pathogens and exogenous antigens. The gut barrier also plays a pivotal role in the regulation of the immune system. The gut-associated lymphoid tissue (GALT) consists of both isolated and aggregated lymphoid follicles, named Peyer's Patches, containing macrophages, dendritic cells (DCs), T- and B-cells [143]. Together with the GALT and the neuroendocrine network, the intestinal epithelial barrier, with its intercellular TJs, safeguards the host from the translocation

of foreign harmful substances and controls the homeostatic balance between tolerance and immunity to non-self antigens. The regulation of this task is achieved by an interplay between structural components and molecular interactions, which operate dynamically to maintain intestinal integrity and immune homeostasis [78-80, 157, 158]. The transport of molecules across the intestinal mucosa occurs through two distinct mechanisms: paracellular diffusion through TJs between adjacent intestinal epithelial cells, and transcellular transport involving the transcytosis of materials often mediated by membrane receptors. The vast majority of absorbed proteins (up to 90%) cross the intestinal barrier through the transcellular pathway. The remaining portion of small molecules, ions, and water-soluble solutes are transported by passive diffusion through the paracellular pathway with a sophisticated regulation of intercellular TJs. [65, 80, 108, 140]. Therefore, the intestinal epithelial layer acts as a physical, biochemical and immunological defense barrier. The alteration of TJs barrier induces an increase of gut permeability, followed by the breaking of the homeostatic equilibrium between the internal and external environments. The gut barrier dysfunction can lead to an uncontrolled flux of dietary or microbe-derived macromolecules across the intestinal epithelium. This may affect the host-microbial balance, and activate the immune system against exogenous antigens. This results, in susceptible individuals, in a final inflammation of the gut. Keys to such dysfunction are genetic, microbial and environmental factors that, singularly or in combination, are putative contributors to the pathogenesis of a spectrum of human diseases including food allergies, CD and inflammatory bowel disease (IBD) [77-79, 80]. In patients with active CD, nine genes related to TJs structure showed altered expression levels [142]. Proinflammatory cytokines, such as TNF-α and others released by DCs, can disrupt proteic components of TJs via phosphorylation and in turn increase the permeability of intestine [76, 77, 140, 153, 155]. Moreover, zonulin, a 47-kDa protein that increases the intestinal permeability, seems upregulated in autoimmune diseases, including CD. In particular, several studies have previously reported that gliadin affects the gut barrier permeability by releasing zonulin. The chemokine receptor CXCR3, which is overexpressed in the acute phase of CD, seems to be the target intestinal receptor for gliadin. The binding of gliadin to CXCR3 is crucial for the release of zonulin from intestinal mucosa and subsequent increase of gut permeability [65, 80]. The gut barrier is not only formed by the epithelial layer but includes other important players. These mainly are the mucus layer with the commensal gut microbiota, antimicrobial peptides (AMPs), and secretory immunoglobulin A (sIgA) molecules released in the mucus layer as immune-sensing and regulatory proteins. [79, 80, 100].

The thickness and formulation of the mucus vary along the different segments of the gut [154]. The mucus layer is the first line of physical defense towards external molecules which arrive in the gut lumen, and it prevents bacteria from directly contacting the epithelial cells. It is in intimate relation

with the columnar enterocytes and goblet cells as well as the innate and adaptive immune systems. The main building blocks of the mucus layer are highly glycosylated mucin proteins that form a gel-like sieve structure overlying the intestinal epithelium. In the small and large intestine, mucin 2 (MUC2) is the main component of the outer mucus layer. It is the most abundant gel-forming glycoprotein secreted by goblet cells. In the outer mucus layer, there are also MUC5AC, MUC6, and MUC5B. Instead, the transmembrane mucins (MUC1, MUC3, MUC4, MUC12, MUC13, MUC15, MUC17, MUC20 and MUC21) cover the apical surface of intestinal cells and form the glycocalyx. They constitute the inner mucus layer which is firmly attached to the epithelial cells [100]. Among all mucins, MUC2 shows pivotal functions and its expression is critical in protection against disease. Indeed, MUC2 not only acts as a physical barrier, but it also provides a stable energy source for the microbiota and enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals (such as IL-10) to dendritic cells (DCs) [144, 145]. The mucus layer is also the interface with the microbiota. Exactly, the outer mucus layer is the natural habitat for the commensal bacteria which preserve the function and structure of the gut barrier and regulate the mucosal immune homeostasis [146-148]. Several data show that the composition of the mucus layer can affect the microbiota in the gut, whilst the microbiota also determines the properties of the mucus gel [79, 101, 144]. Thus, there is a mutual interaction between the mucus and the microbiota [154]. In particular, mucins can act as an environmental cue for bacteria to modulate the expression of virulence/colonization related genes. On the other hand, bacteria have mucus stimulating capacity by using small compounds, post-biotics, that diffuse over the inner mucus layer [144, 145, 150, 154]. Certain bacteria can, in contrast to promoting well-developed mucus, also destroy the mucus and its protective properties. Thus, immune regulators, AMPs such as defensins and lysozyme, and sIgA molecules are distributed in the mucus gel to reinforce the physical separation between the microbiota and the epithelium and contrast the injuries from pathogens. These substances show the highest concentrations in the small intestine where the mucus layer is less dense [101, 144]. Furthermore, both the mucus layer and microbiota are highly connected to the immune system as they are fundamental players in the establishment of immune tolerance to food and commensal bacteria antigens [145]. Bacteria and their community structure affect epithelial barrier properties and mucus secretion in ways that can have implications for health and disease, but molecular mechanisms are far from understood. We are just at the beginning of answering fundamental questions concerning the diverse repertoire of goblet cells and different mucus components. Even more important is to understand the signaling systems connecting the intestinal microbiota, epithelium, and the immune systems related to intestinal inflammation [101, 144].

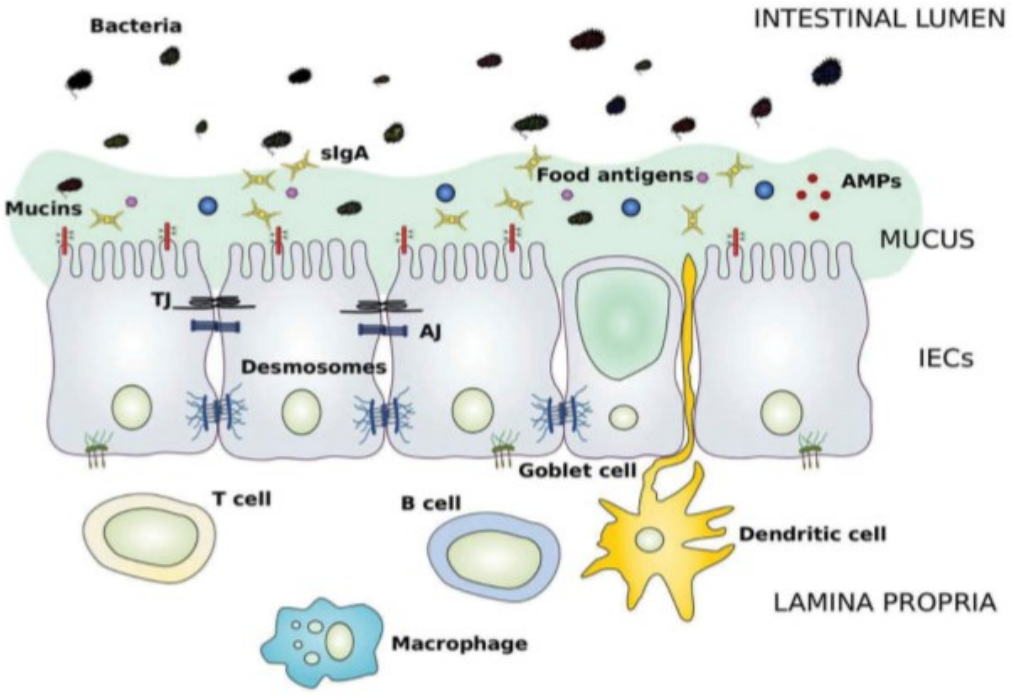

Fig. 1.10. Schematic representation of the main components of the intestinal barrier.
The intestinal barrier is a semipermeable structure. Both structural and molecular components act together to fulfill this complex, but essential function of the gastrointestinal tract. The mucus layer forms a sieve-like structure overlying the intestinal epithelium. Antimicrobial peptides (AMPs) and secretory IgA molecules (sIgA) are secreted in the mucus layer as immune-sensing and regulatory proteins. The intestinal epithelial cells (IECs) form a continuous monolayer and are tightly attached to each other by junctional complexes. The tight junctions (TJs) are located at the apical side of the cells and regulate the transport of small molecules and ions. The adherens junctions (AJs) and desmosomes provide strict cell-adhesion bonds and aid in the maintenance of the integrity of the intestinal barrier. The *lamina propria* contains immune cells (e.g. T cells, B cells, macrophages, and dendritic cells) from the adaptive and innate immune system that take part in the immunological defense mechanisms of the intestinal barrier [79].

3.3.3 Role of the microbiota in gut homeostasis and celiac disease

Intestinal microbiota plays an important role in normal gut function, providing beneficial effects to the host [92, 146, 147]. Thus, the host and microbes are in a state of symbiotic mutualism. The composition of the microbiota is unique and reaches up to a density of 1×10^{12} bacterial cells per gram of content in the adult colon. The composition and density vary along the length of the intestine as well as with age. These microbes are classified into largely four different phyla: *Firmicutes, Bacteroidetes, Proteobacteria*, and *Actinobacteria* [75, 78, 114, 131]. Specifically, gut hosts about 30 species of *Bifidobacteria*, 52 of *Lactobacillus,* others such as *Streptococcus, Enterococcus* and also yeast such as *Saccharomyces.* The genes of microbiota (microbiome) outnumber those in the human genome by approximately 100-fold. The microbiota colonizing the gut after birth reaches the pattern found in adults within 2-3 years of life [131, 147, 149, 159]. Genetic and environmental factors are known to influence the composition of the gut microbiota and increase the interpersonal variability

[75, 131,162]. Among all microorganisms that inhabit the gastrointestinal tract, probiotics have beneficial effects. Available evidence indicate that they release a series of metabolic products, post-biotics, which help the host with maturation and homeostasis of immune system, nutritional assistance, growth and apoptosis of intestinal epithelial cells, protection of mucosal barrier function, mucins secretion, prevention of injurious effects caused by xenobiotics and pathogens, brain development and behavior [146-149, 154, 156]. Especially, several mechanisms appear to be associated with the mucosal protective role of probiotics from a variety of insults including infection by pathogens. Probiotics, such as several strains of *Lactobacillus* and *Bifidobacterium,* interfere with the adhesion of gastrointestinal pathogens by steric hindrance and competitive exclusion. Signals from the microbiota induce the development of various innate lymphoid cell subsets and production of IL-22 which in turn stimulates the release of AMPs such as RegIIIγ, from Paneth cells, γδTCR$^+$ IELs and epithelial cells. Microbiota also stabilizes cellular cytoskeleton and gut barrier permeability to prevent epithelial invasion. The barrier protective effect involves the release of metabolic molecules, which in turn regulate the TJs integrity and, indirectly, villi architecture [92, 131]. Furthermore, the differentiation of B cells to IgA-producing plasma cells is also dependent on the microbiota.

The microbiota releases short chain fatty acids (SCFAs, principally referring to acetate, propionate and butyrate) which have a pivotal role in gut health. Indeed, SCFAs enhance the intestinal epithelial barrier by regulating the expression, the assembly of TJs proteins and prevent their disruption [75, 146, 156]. They also modulate macrophage and DCs function, anti-inflammatory cytokine secretion, Treg differentiation, intestinal goblet cell mucin secretion and neutrophilchemotaxis [131, 150-152]. In particular, butyrate has been reported to have immunomodulatory effects on intestinal macrophages and thereby conferring them hyporesponsiveness to commensal microbiota. Apart from the predominant energy source for the colonocytes and enterocytes, butyrate has anti-inflammatory and anti-cancer activities. Furthermore, probiotics also produce polyphosphates that enhance the gut barrier function, suppressing oxidative stress, and maintain intestinal homeostasis by an integrin-p38 MAP kinase-dependent mechanism. In the colonic crypts, resident bacteria release compounds such as muramyl dipeptide, which contribute to stem cell survival and favor epithelial cell regeneration during the oxidative stress [150-154].

The digestive tract is in direct contact with foreign antigens and microorganisms. While a strong and protective response is required to eliminate pathogens, tolerance is essential for harmless antigens or nutrients [75, 146]. The ability of the immune system to keep tolerance to commensals while remaining capable of responding to injury or infections is essential for tissue homeostasis. Moreover, exposure to commensal bacteria is essential for normal immune development and its absence can lead

to long-term effects on immune development [147-149]. The composition and diversity of gut microbiota are fundamental to maintain the homeostasis of the human body [92, 159-161]. Several host immune-regulatory mechanisms have evolved to prevent inappropriate activation of inflammatory response to the commensal flora and maintain the native microbiota [147, 148]. Thus, resident macrophages show hyporesponsiveness to Toll-like receptor ligands of commensal bacteria [75]. Furthermore, sIgA neutralize the bacteria so that they cannot enter the host by themselves, but at the same time allow a controlled sampling of the microbes for further production of specific IgA molecules. The sIgA are also involved in allowing binding and colonization of commensal bacteria to the mucus and maintenance later in life [147, 148, 154]. Indeed, most of the bacteria are coated with IgAs in the small intestine. This kind of mucosal antibodies allow microbiota diversification, maintenance, and life-long persistence. The IgA absence could allow the expansion of bacterial strains such as y-proteobacteria or segmented fifilamentous bacteria [154]. Besides, it is well accepted that clonal deletion and/or T cell anergy are components of the mechanism of action of oral tolerance, but Treg induction has become widely known as its central component. The predominant mechanism by which $CD103^{+}$DCs induce and maintain peripheral tolerance involves the generation of $CD4^{+}FOXP3^{+}$ Tregs from naïve T cells [75, 104]. The microbiota with its products contribute to the development of Treg cells and immune tolerance, however, the molecular mechanism remains unclear [150-152]. Several commensal *Bacteroides* and *Bifidobacteria* strains can directly induce monocyte-derived DCs to acquire a tolerogenic phenotype and drive a mixture of Th1 systemic responses and IL-10 producing $CD4^{+}FOXP3^{+}$ Treg cells. The $CD103^{+}$ DCs maturation and antigen presentation can be tolerogenic or immunogenic dictated by the microenvironment [92, 148-150]. Under steady-state conditions, lamina propria-resident $CD103^{+}$ DCs are tolerogenic (Fig. 1.11a). Generally, induction of immunity versus tolerance by intestinal DCs is mediated by phatogenic microbial stimuli and proinflammatory cytokines [105-107].

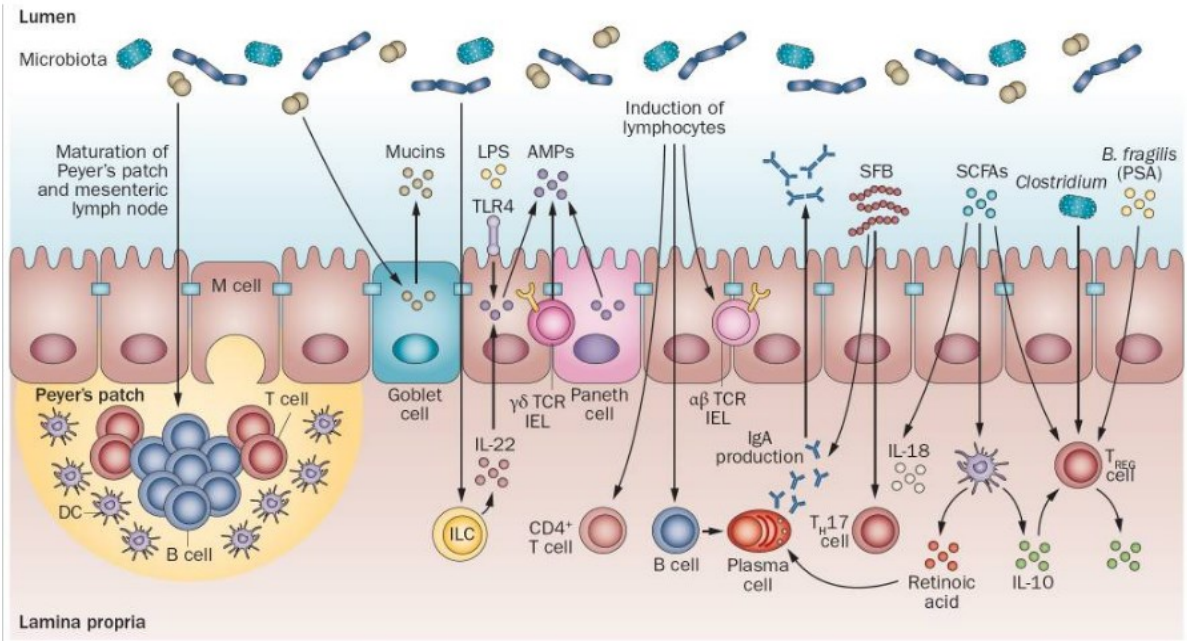

Fig. 1.11a. Gut microbiota shapes host immunity. The gut microbiota induces maturation of the gastrointestinal lymphoid tissue, AMPs production, mucin secretion and the development of T cell subsets which are critical for the induction of IgA-producing plasma cells. SCFAs stimulate Treg cell differentiation. They also promote IL-18 production from epithelial cells, IL-10 and retinoic acid release from DCs, which in turn promote the differentiation of T_{REG} cells and IgA-producing plasma cells [131].

Although the intestinal tract is colonized by large number of commensal bacteria, it seems possible that some species take over and generate a situation of dysbiosis. Intestinal dysbiosis reached through caesarian section, formula-based diet, hygiene, vaccinations, and use of antimicrobials in infants, alters the outcome of immune development [147, 149, 160, 161]. Furthermore, it potentially induces the translocation of bacterial antigens that predispose individuals to intestinal infections, inflammatory and metabolic diseases such as diabetes and obesity [78, 114]. Intestinal dysbiosis leads to impairment of oral tolerance and it seems to be associated with CD development [131]. In particular, the loss of tolerance allows the interaction of some commensal bacteria with certain intracellular PRRs, namely nucleotide-binding oligomerization domain (NOD)-like receptors, activates the release of pro-inflammatory cytokines and free radicals that may cause TJs damage and increase gut permeability [159]. Consequently, dietary gluten peptides reach the *lamina propria* and activate DCs with a proinflammatory phenotype that induce an aberrant Th1-mediated response [92,114,159,162]. Molecular techniques have shown that, compared to the fecal and duodenal microbiota of healthy individuals, the fecal and duodenal microbiota of CD patients is characterized by the presence of higher number of gram-negative bacteria (*Bacteroides* and *Enterobacteria*) and lower number of gram-positive bacteria (*Lactobacillus* and *Bifidobacteria)*. The number of bacteria of *Streptococcus* and *Prevotella* genera were found to be lower both in adults and children with untreated CD in comparison with healthy controls [92, 120, 126, 159**]**.

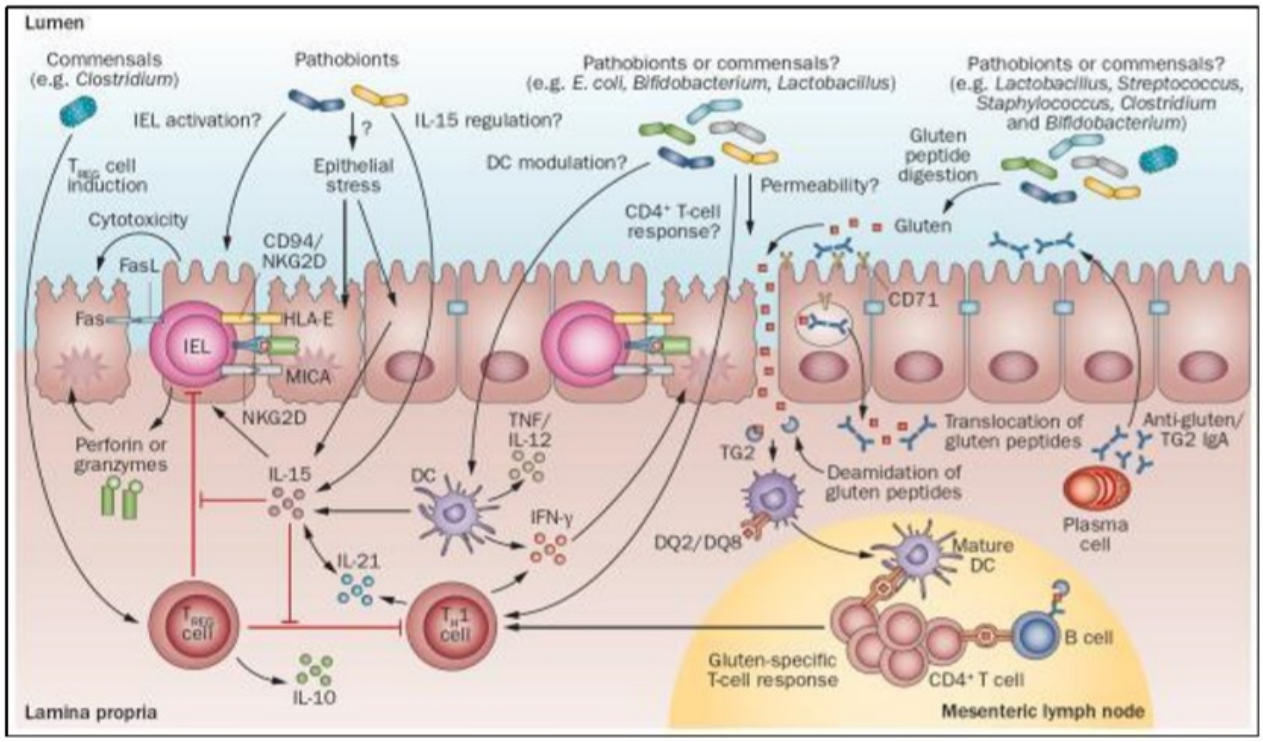

Fig. 1.11b. Potential microbial modulation of coeliac disease pathogenesis. Microbes might contribute to the development of CD by influencing Treg cell induction, epithelial cell stress, IELs activation or upregulation, IL-15 regulation, DCs maturation and proinflammatory cytokines production, intestinal permeability modulation, gluten peptide digestion, and induction of $CD4^+$ Tcell responses [131].

Overall, studies suggest that the induction of immune response by the gut microbiota is influenced not only by the presence or absence of live bacteria (germ-free versus colonized conditions) but also by the relative abundance of particular members of the microbiota and their by-products [131]. Moreover, the exact mechanisms through which the gut microbiota might influence the CD onset or progression is unknown but could include alteration of the gut barrier and mucus secretion, activation of innate immune system with proinflammatory cytokines release and exacerbation of the gliadin-specific immune response in genetically susceptible individuals (Fig. 1.11b).

4. Clinical features and diagnosis of celiac disease

The clinical spectrum of CD is wide and holds a variety of symptoms and histological lesions [77, 80, 96]. Classical CD is characterized by clinically evident gastrointestinal signs and symptoms (malabsorption, diarrhoea, steatorrhoea, abdominal pain, bloating) attributable to gluten intake. Classic CD is frequently found in conjunction with autoimmune diseases, such as type 1 diabetes mellitus or autoimmune thyroiditis. Patients with long-standing untreated classical CD are at risk for developing enteropathy-associated T cell lymphoma and other cancers of the gastrointestinal tract [68]. Non-classical CD has been used to describe patients with gluten-induced enteropathy and extra-

intestinal manifestations (chronic fatigue, headache, osteoporosis, anemia, depression). Due to atypical features, many CD cases currently escape diagnosis and are exposed to the risk of long-term complications. Individuals with asymptomatic or subclinical CD do not manifest any symptoms commonly associated with CD and have no symptoms that respond to gluten withdrawal. Many of these patients suffer from decreased quality of life [61, 62]. Instead, refractory CD shows recurrent malabsorptive symptoms and signs with villus atrophy. It develops in a small percentage of adult patients (25%) and despite strict adherence to gluten-free diet for more 12 months, there is a significant rise in IELs which can develop to T-cell lymphoma [62,76].

Typically, the histopathological features of CD include partial to total villous atrophy with a villous: crypt depth ratio of < 2, and an increased IELs count (most often > 25/100 enterocytes) [77, 96, 99, 103]. The varying degrees of villous atrophy is graded according to a histological classification system proposed by Marsh and modified by Oberhuber (Marsh I-IIIc grade) [61, 64,113]. Furthermore, from a serological point of view the presence of specific endomysial IgA antibodies (EMA), anti-tissue transglutaminase antibodies (tTG), and/or deamidated antigliadin antibodies (IgG class) plays an important role in CD. These antibodies strongly support the diagnosis of CD, but by themselves are not confirmatory. Indeed, high serum levels of anti-tTG IgA and anti-EMA IgA identify individuals who need the intestinal mucosa biopsy to confirm CD diagnosis. [76, 80, 83]. Clinicopathological correlation is key to the diagnosis of CD in adults and children. Confirmation of the diagnosis by duodenal biopsy is the gold standard in adults [61, 62, 77]. In particular, duodenal biopsies must be taken when patients are on a gluten-containing diet. The consensus states four to six biopsies are necessary for diagnosis, including from the duodenal bulb [61, 64].

According to the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) guidelines, in adolescents and children with symptoms or signs suggestive of CD and with high anti-tTG levels (>10 times the normal upper limit), positivity for anti-EMA antibodies and HLA-DQ2 or HLA-DQ8 heterodimer, the duodenal biopsy can be avoided because the likelihood for villous atrophy (Marsh III classification) is high. The wide variability of CD-related findings suggests that it is difficult to conceptualize the diagnostic process into rigid algorithms that can cover the clinical complexity of this disease. For this reason, a quantitative approach that can be defined as the 'four out of five rule' was proposed recently. Using this method, the diagnosis of CD is confirmed if at least four of the following five criteria are fulfilled: 1) Typical symptoms of CD. 2) Positivity of serum CD IgA class autoantibodies at high titer. 3) HLA-DQ2 and/or HLA-DQ8 genotypes. 4) Celiac enteropathy found on small bowel biopsy. 5) Response to a gluten free diet [96]. However, despite significant changes in the CD diagnosis due to the steady increase in the number of diagnoses identified, a screening test that totally avoids an invasive approach is still not known [82, 125].

Numerous researches have tried to find novel molecular biomarkers for the diagnosis or follow up of the intestinal damage, or to find alternative methods to avoid invasive biopsies. In the last two decades, a class of small non-coding RNA, called microRNAs (miRNAs), have been widely studied and discovered to regulate many biological processes such as differentiation, development, and cell death. In patients affected by CD, the expression of specific miRNAs is altered, suggesting that they could be involved in the pathogenesis of disease and be associated with particular clinical features [77]. Also, the identification of genes playing an important role in CD will ultimately open new ways for disease diagnosis, prognosis, prevention, and therapy [91].

5. Potential therapeutic approaches in celiac disease

Gluten free diet is currently the only therapy available for patients with CD. While effective, it is difficult to maintain due to cost, availability of products, social acceptance. Given these obstacles, as many as 50% of patients with CD who adhere to a gluten free diet do not achieve histologic remission [62]. Thus, several efforts have been made to explore potential therapeutic approaches for celiac patients. The emerging area of cellular therapy for CD is mainly based on stem cell therapy which has the advantage of targeting multiple pathways and has yielded promising results. It is crucial to bear in mind that the intestinal tract has a highly regulated process for regeneration mainly due to the harsh environment which it is exposed. All the differentiated epithelial cells of the intestine derived from a single intestinal stem cell (ISC) (CD133+/Lgr5+ crypt cell) compartment which resides at the crypt base. The amplifying cells that are generated from ISC migrate upward and become differentiated villous epithelial cells. Hematopoietic stem cell (HSC) transplantation could be an effective treatment for patients with severe refractory CD compare to conventional treatments like an ineffective gluten free diet. The HSC transplantation has shown strong modulatory effects on all immune cells together with a potential regenerative effect [76].

Furthermore, knowing that microbiota and some parasites can affect intestinal permeability and exert an immunomodulatory effect, there is increased interest in the use of probiotics and helminths as possible therapeutics for CD. Especially, probiotic therapy can ameliorate the inflammation induced by gluten and be useful as supportive therapy for the gluten free diet [146]. Instead, helminths are safe and capable of suppressing the immune responsiveness of celiac patients when exposed to gluten [97]. Fecal microbiota transplantation could be another therapeutic approach for the treatment of recurrent gastrointestinal and non-gastrointestinal disorders [147, 148]. Indeed, it could quickly reestablish a normal microbiota, with the restoring dominance of *Bacteroides* and *Firmicutes* in the distal gut [193]. Besides, TG2 inhibitors and Larazotide are therapeutics that aim to maintain the integrity of TJs as a way of preventing downstream inflammatory cascades. TG2 inhibitors are also

effective in reducing the production of anti-tTG antibodies and corresponding crypt cell proliferation. Another method of treating CD is to target different key molecules in the inflammatory cascade, such as cytokines. Indeed, antibodies targeting the IL-15 receptor or IL-15 itself were successful in inhibiting villous atrophy [97]. However, due to the complex cascades of immunological pathways in CD, the newly developed therapies often have unsatisfactory effects, mainly because they tend to target a single pathway instead of the modification of multiple mechanisms. Thus, the development of an alternative cure is still a long way off [76, 91].

6. Gluten-Free Diet

The gluten proteins contained in wheat flour are crucial during the bread and pasta making process since they confer the viscosity and elasticity to the dough. Besides, wheat flour provides foodstuffs important nutritional components, including dietary fiber, vitamins, and minerals.

Although the amounts of gluten ingested might vary considerably from individual to individual, depending on diet choices, gluten proteins are responsible for a common and severe intolerance in a large number of individuals [38, 174-177]. To date, the first-line and the only safe and efficacious treatment for people with CD is a life-long strict exclusion of gluten, the so-called gluten-free diet (GFD) [171, 175]. GFD spread in the early 1990s and universally changed the food culture. The replacement of the unique technological properties of wheat gluten represents the major task of the industry for providing high-quality gluten-free foods in terms of structure, loss of starch during cooking, and optimal cooking time. Gluten-free now constitutes a major food industry due to its popularity among not only celiac patients but also individuals with other gluten-related conditions. This is also due to the improvements obtained in both the range of foods available and their overall palatability [38, 172]. Although GFD brings great benefits in the majority of celiac individuals, adhering to it is difficult because gluten is present in a large number of foodstuffs. The foods not allowed in the GFD include: all types of bread and food prepared with wheat flour, including kamut, spelt, rye, barley, triticale, or with components from these flours; food that contains wheat, or derivatives of gluten used as thickeners, such as hot dogs, salad dressing, sauces, some types of cheese and cold cuts; medicinal products that use gluten as binder in the pills or tablets [38, 171]. Furthermore, gluten is not totally absent even in the so-called gluten-free industrially processed foods. Cross-contaminations might occur in the production line when the gluten-free products share the same facilities and/or equipment with gluten-containing items. The dietary transgression of GFD is a major factor for refractory symptoms and persistently abnormal histology in celiac patients. For this reason, an ongoing regulation and control of certified gluten-free foods are mandatory [172, 175, 176].

Nowadays, gluten cannot be hidden in foods as allergen labeling was introduced in the European Union (EU) in 2005. Now, all wheat, rye, barley, and oat ingredients must be listed in the ingredients list. The amount of gluten capable of initiating an antigenic reaction has been estimated to be > 20 mg/Kg (or part per millions, ppm) of gluten, and contamination below 20 ppm is considered safe over a wide range of foods in daily consumption. In particular, the EU gluten-free legislation published in 2009 and regulated in 2012 specifies two levels: gluten-free (< 20 ppm) and low gluten (20-100 ppm), but in practice, the only gluten-free standard is applied. If products do not have any gluten-containing ingredients, then an associated threshold would not be necessary [172]. However, cross-contaminations of gluten-free foods cannot be entirely avoided because some of them might occur also during the time of cooking gluten-free foods at home, or when eating out or when consuming ready-to-eat foods or due to any potential source of gluten present in kitchens [175, 176]. The tolerable daily intake level for gluten in individuals with CD was calculated at 0.4 mg gluten/day for adverse morphological effects and at 0.015 mg gluten/day for adverse clinical effects, even if highly variable gluten sensitivity is observed among patients [175].

Though from a nutritional point of view, gluten exclusion might not involve particular problems, being a mixture of proteins with low nutritional and biological value, the GFD creates enormous limitations, above all in the family, social and working contexts related to food, deteriorating the quality of life [171, 175]. Gluten-free products also exhibit poor mouth fell or flavor and, no less important, are particularly expensive. In addition, GFD may not guarantee an adequate nutritional intake, in fact the 20–38% of celiac patients experience nutritional deficiencies. It is unclear if the persistent malabsorption syndrome accounts for some part of these deficiencies [38, 172]. GFD has often low content of vitamins and ions, such as water-soluble vitamins B (B_6, B_9, B_{12}) and fat-soluble vitamin D, calcium, iron, zinc, and magnesium, as well as fiber. The vitamin B_{12} deficiency and macrocytic anemia, usually caused by folate (B_9) deficiency, are often observed in untreated celiac patients [38, 171]. Instead, the vitamin D deficiency, which gives rise to the osteomalacia, is usually due to secondary lactose intolerance in celiac patients [171, 174]. Furthermore, gluten-free foods prepared with corn and rice starch have a high glycemic index that increases the risk to develop metabolic syndromes in celiac patients. The use of palm oil, cream and high-fat powder aimed to improve the palatability of cereal-based gluten-free products, renders these products highly caloric. It is well known from epidemiological studies that the daily consumption of high glycemic index food is correlated with the risk to develop cardiovascular disease, obesity, and diabetes [38]. Previous studies suggest that celiac patients are at risk of overweight/obesity especially during the first year after starting the GFD; probably also influenced by the fact that they can eat without suffering symptoms and feeling ill [175]. Another important aspect related to dietary restriction is the alteration

of gut microbiota composition. It might be due to functional and structural changes of the intestinal tract and to the low content of fiber in the GFD [171, 173]. Nevertheless, in recent years, interest in GFD has greatly increased and many people follow it also in the absence of CD or other conditions that justify it. Today, three conditions that require treatment with GFD are identified: a) CD; b) a new condition referred to as non-celiac gluten sensitivity (NCGS), includes individuals who report symptoms that respond to withdrawal of gluten from the diet in the absence of CD, and c) wheat allergy [175]. The GFD should be prescribed only once CD diagnosis has been established using serology and duodenal histology. In potential CD, the diet should be reserved for subjects reporting symptoms, while those asymptomatic can be maintained on a gluten-containing diet but should be followed up regularly. In patients with symptomatic CD, the diet should be followed strictly also in view of the risk for complications.

Strict adherence to a GFD has been shown to lead to partial, if not complete, healing of the duodenal mucosa along with the resolution of clinical symptoms and signs of malabsorption in the vast majority of celiac patients. It has been observed that mucosal recovery takes more time and is more frequently incomplete in adults than in children. In a few cases, despite the clinical symptoms decline, mucosal abnormalities may persist. The intraepithelial lymphocytosis persisting in CD patients during GFD cannot completely be eliminated and is independent of the duration of adherence to a standard GFD. In line with these findings, clinical improvement of symptoms was not a reliable marker of mucosal recovery. Initially, it was also hypothesized that strict adherence to GFD could prevent the development of further autoimmune diseases and CD-related complications (e.g., refractory disease, intestinal lymphoma) in celiac patients, but current data allow for the assumption that the GFD has no definite role in the prevention of development of other autoimmune conditions. Furthermore, the outcome of GFD within the subgroups of asymptomatic and potential CD is mostly unclear. There remain, however, open issues regarding not only the tolerable threshold of gluten ingestion but also the appropriateness of a lifelong indication to GFD. Since the natural course of CD is not clearly understood, it is still unclear if a strict GFD needs to be followed throughout the whole life in all patients or if tolerance may develop in certain patients [176].

Despite extensive research aimed at developing alternative therapies for CD, the GFD remains the only effective treatment available to date.

6.1 Advances in the formulation of Gluten-Free Food

Being a protein of low nutritional value, gluten is massively used by food industries. In addition to the high glycemic index and caloric density of cereal-based gluten-free food, these products generally are not enriched/fortified, so that, they may not contain the same levels of nutrients, as the natural

wheat grains. For these reasons, several studies investigated the preparation of a new generation of cereal-based gluten-free food. The enrichment of baked products with dietary fibers and devoid of gluten has been the goal of the recent years [38, 175]. Inulin is the most acceptable dietary fiber and acts as a source of non-digestible polysaccharides and prebiotics in gluten-free products. As a prebiotic, inulin stimulates the growth of healthy bacteria in the colon. Also dairy protein, with low lactose, has also been incorporated into the baking industry for improving nutritional and functional quality along with flavor, texture, and storage time of products. Indeed, after incorporating dairy-based protein, the handling properties of the batter are enhanced because of increased water absorption. Nevertheless, precaution should still be taken regarding the incorporation of lactose-rich powders during formulation of gluten-free breads for celiacs, because the damaged intestinal villi fail to produce lactase enzyme and, consequently, lactose intolerance could be noticed among those patients [182]. Among the novel approaches that search for improving nutritional qualities of gluten-free products, it is worth mentioning gluten-free bread with quinoa and flaxseed, which have a better balance of polyunsaturated/saturated fatty acid and would supply low levels of trans-fatty acids, with good acceptance. Quinoa and flaxseed are also recommended to improve the amount of ω-3 fatty acids in gluten-free products [97,179]. Processed foods based on the pseudocereals amaranth, quinoa, sorghum and buckwheat are rich in proteins with high biological value (albumins and globulins), fat, fiber and minerals in comparison with those based on rice and corn, becoming good alternative ingredients for gluten-free products [175, 178, 182].

7. Methods of detoxification of Wheat Gluten

Patients find the GFD to be exceedingly burdensome because it is socially restrictive and more expensive than ordinary food. For these reasons, there is a need for alternative treatments of CD, as suggested by the intensive research efforts undertaken in different laboratories [172].

The endeavors to genetically modify grains to diminish the immunogenic components have been started years ago. Genetically-modified wheat is a promising area of research, where successful attempts have been made to silence the gliadin gene of wheat using RNAi techniques. Recently, some studies have tried to edit the α-gliadin genes containing the 33-mer immunodominant epitope by using CRISPR/Cas9 technology [182-184]. Numerous studies in food technology are currently devoted to prepare pasta and baked goods made from wheat flours modified in order to eliminate, or reduce, the immune toxicity of gluten proteins (detoxification process). The first method, using endopeptidase of bacterial origin during the preparation of wheat flour dough, results in the complete degradation of gluten peptides including those that are strongly immune toxic for celiacs. Such an approach, carrying

out total destruction of the gluten network, reduces the technological properties (viscoelasticity) of dough and, consequently, of pasta or baked goods, unless the flour is integrated with structuring agents, as pre-gelatinized starches (i.e. maize, potato, rice), emulsifiers (i.e. lecithins) or hydrocolloids (i.e. guar and xanthan gums, alginate) [38, 175, 182]. Digestion of dietary gluten has also been proposed based on administering oral endopeptidases derived from plants, bacteria or fungi that can hydrolyze gluten peptides along the digestive tract, but the remnant gluten contained in these products and the degree of digestion reached remain uncertain [97,175, 180]. Another method to detoxify gluten proteins uses the specific transamidation of toxic epitopes done by the tissue-transglutaminase of microbial origin (Streptomyces mobaraensis) in the presence of lysine methyl ester. This method has the great advantage of blocking the immunogenicity of T cell epitopes (as demonstrated in an *in vitro* assays using intestinal T cell from celiacs), and more importantly, it keeps intact the gluten network and preserves the technological properties of the flour. Furthermore, this procedure uses an enzyme largely employed in the food industry for improving the texture of foods. A preliminary 90-days trial made with CD subjects in remission consuming bread slices with transamidated gluten indicated that only a subgroup of celiacs exhibited clinical symptoms compared to subjects consuming the toxic gluten. The researchers have now implemented the transamidation reaction in order to reach protection in the great majority of CD volunteers that eat detoxified wheat flour [38, 181].

8. Gluten Friendly ™ Technology

In recent years, a novel temperature-based method called "Gluten Friendly™" has been developed. This is an Italian patented method n°: 0001414717, also filed under the Patent Cooperation Treaty, application no. PCT/IB2013/000797 and published in Europe as EP 2903453 A1 and titled "Detoxification method of gluten proteins from cereal grains" [185, 186]. Gluten Friendly ™ technology allows the reshaping of gluten proteins directly in the cereal grains before milling. In particular, Gluten Friendly ™ method relies on the application of high temperature cycles for a short amount of time (reached by microwave energy), alternated with evaporation and ventilation phases, applied to the wheat kernels before milling. This is a wise combination of parameters such as temperature, humidity, time of rest and evaporation, clearly described in the registered patent. The beginnings of Gluten Friendly™ technology relies on a previous study in which Lamacchia et al. [187], reported that the high temperatures applied to wheat kernels induced significant structural changes in proteins but these were different from those reported in a gluten model system and pasta. The explanation for this phenomenon is that, in the kernels, gluten is not yet formed and gluten proteins are deposited in different protein bodies [187], as confirmed by Tosi et al., in a following

study [59]. In particular, Gluten Friendly ™ method reshapes the tertiary structure of gluten proteins, when they are in protein bodies inside the starchy endosperm of wheat kernels, making them unrecognizable by gluten-specific antibodies *in vitro.* While the temperature-treated gluten loses its immunogenic properties *in vitro*, it does not lose its technological properties and viscoelasticity (its ability to create a matrix and form dough) [188]. A previous *in vitro* study carried out on wheat kernels treated with Gluten Friendly™ technology showed a reduced cross-reactivity of antibodies recognizing almost the entire range of gluten proteins due to conformational changes of gluten protein structure in the kernel [189]. Furthermore, in the recent years *in vitro* studies have been performed to evaluate the effects of Gluten Friendly bread (GFB) compared to control bread (CB) on the microbiota of celiac patients and healthy controls. The results obtained showed that in 48 hours the GFB, unlike CB, restored the composition of intestinal microbiota in celiac patients, prolonged the survival of the probiotic *L. acidophilus* (La-5) and exerted a bifidogenic effect. GFB was also able to exert *in vitro* an antibacterial effect against pathogens such as gram-negative *S. Typhimurium* and gram-positive *S. aureus* [190]. These results were confirmed in a following *in vitro* study, using a gut model system that was formed by three different vessels connected in series. Each vessel mimed the proximal, transverse, and distal tract of the human colon. The digested GFB and CB were incubated, in the gut model, with fecal homogenate from healthy individuals and celiac patients for a prolonged period of 30 days. At the end of the study, the effective restoration of the intestinal microbiota was confirmed not only in celiac volunteers but also in healthy subjects, which showed an increased number in bifidogenic bacteria and SCFAs levels, mainly propionic and butyric acid [191].

The results obtained from these recent studies clearly show that the Gluten Friendly™ molecule acts as a probiotic booster with bioactive properties. Furthermore, this gives the way to obtain, from wheat kernels, functional flour with beneficial effects for gut health. Based on this evidence, the technology has been further improved with the registration of a second patent: "Method for the detoxification of gluten proteins from cereal grains and related uses in the medical field" (Italian priority patent no: 102015000084813 also extended to European level, PCT/EP2016/0815899) (Inventor: Lamacchia C.) [192].

References

1. **WheatBP** (online), http://www.cerealsdb.uk.net/.
2. **Gramene**. Species (online), http://www.gramene.org/.
3. **Dinu, M.**, Whittaker, A., Pagliai, G., Benedettelli, S., Francesco, S. (2017). Ancient wheat species and human health: biochemical and clinical implications. *The Journal of Nutritional Biochemistry,* doi: 10.1016/j.jnutbio.2017.09.001.
4. **Shewry, P. R.** (2009). Wheat. *Journal of Experimental Botany*, Vol. 60, No. 6, pp. 1537–1553.
5. **Feldman, M**. (2001). Origin of cultivated wheat. *The world wheat book: a history of wheat breeding*. Paris, France: Lavoisier Publishing, 3–56.
6. **FAO** (online), http://www.fao.org/3/y4011e/y4011e04.htm.
7. **Mayer, KFX.** (2014). A chromosome-based draft sequence of the hexaploid bread wheat (Triticum aestivum) genome. *Science*, 345:1251788.
8. **Sands, D. C.**, Morris, C. E., Dratz, E. A., Pilgeram, A. (2009). Elevating optimal human nutrition to a central goal of plant breeding and production of plant-based foods. *Plant Science*, 117(5): 377–389.
9. **Wikipedia**. Wheat. https://en.wikipedia.org/wiki/Wheat.
10. **Delcour, J. A.**, Joye, I.J., Pareyt, B., Wilderjans, E., Brijs, K., and Lagrain, B. (2012). Wheat Gluten Functionality as a Quality Determinant in Cereal-Based Food Products. *Annual Review of Food Science and Technology*, 3:469–92.
11. **Rasheed, A**., Mujeeb-Kazi, A., Chuks Ogbonnaya, F., He, Z. and Rajaram, S. (2017). Wheat genetic resources in the post-genomics era: promise and challenges. *Annals of Botany*,121: 603–616.
12. **Allen, A.M.**, Winfield, M. O., Burridge, A. J., Downie, R. C., Benbow, H. R., Barker, G. L. A., Wilkinson, P.A., et al. (2017). Characterization of a Wheat Breeders' Array suitable for high-throughput SNP genotyping of global accessions of hexaploid bread wheat (Triticum aestivum). *Plant Biotechnology Journal,* 15, 390–401.
13. **Gianibelli, M. C.**, Larroque, O. R., MacRitchie, F., and Wrigley, C. W. (2001). Biochemical, Genetic, and Molecular Characterization of Wheat Glutenin and Its Component Subunits. *Cereal Chemistry*, 78(6):635–646.
14. **Bhalla, Prem L.** and Singh, Mohan B. (eds.). (2017). Enabling Molecular Technologies for Trait Improvement in Wheat. *Wheat Biotechnology: Methods and Protocols, Methods in Molecular Biology,* vol. 1679*,* chapter 1.
15. **Dhanavath, S.** and Prasada Rao, U.J.S. (2017). Nutritional and Nutraceutical Properties of Triticum dicoccum Wheat and Its Health Benefits: An Overview. *Journal of Food Science,* 82 (10):2243-2250.
16. **Šramkováa, Z.**, Gregováb, E., Šturdíka, E. (2009). Chemical composition and nutritional quality of wheat grain. *Acta Chimica Slovaca*, Vol.2, No.1, 115 – 138.
17. **Belderok, B.**, Mesdag, H., Donner, D.A. (2000) Bread-Making Quality of Wheat. *Springer*, New York.
18. **Liu, Y.**, Zhang, L., Li, Y., Yang, Y., Yang, F., Wang, S. (2018). The Functional Properties and Structural Characteristics of Deamidated and Succinylated Wheat Gluten. *International Journal of Biological Macromolecules,* Vol. 109, 417-423.
19. **Žilić, Slađana**. (2013). Wheat Gluten: Composition and Health Effects. *Gluten: Sources, Composition and Health Effects*, Dane B. Walter Editors, Chapter 4, 71-86.
20. **Shewry, P. R.** & Hey, S. J. (2015). The contribution of wheat to human diet and health. *Food and Energy Security*, 4 (3): 178–202.
21. **Tatham, A. S.**, Shewry, P. R. (1985). The conformation of wheat gluten proteins. The secondary structures and thermal stabilities of α-, β-, γ- and ω-Gliadins. *Journal of Cereal Science,* Vol. 3, 103-113.
22. **Khatar, B.S.**, Schofield, J.D. (1997). Molecular and physico-chemical basis of breadmaking-proprieties of wheat gluten proteins: a critical appraisal. *Journal of Food Science and Technology,*34, 85-102.
23. **Shewry, P. R.** and Tatham, A. S. (1990). The prolamin storage proteins of cereal seeds: structure and evolution. *Biochemical Journal*, 267, 1-12.
24. **Egorov, T. A.**, Odintsova, T. I., and Musolyamov, A. K. (1999). Determination of Disulfide Bonds in γ-46 Gliadin. *BIOCHEMISTRY* (Moscow), Vol. 64, No. 3, 294-297.
25. **Khatkar, B. S**. (2014). Structure and functionality of wheat gluten proteins. *International Conference of Food Properties (ICFP)*.
26. **Shewry, P.R.** (1992). Exploring the Structure and Functionality of Wheat Gluten Proteins. *Biotechnology & Biotechnological Equipment*, 6:2, 5-10.

27. **Merlino, M.**, Leroy, P., Chambon, C., Branlard, G. (2009). Mapping and proteomic analysis of albumin and globulin proteins in hexaploid wheat kernels (Triticum aestivum L.). *Theoretical and Applied Genetics*, Vol. 118, 1321–1337.
28. **Tomić, J.**, Torbica, A., Popović, L., Strelec, I., Vaštag, Ž., Pojić, M., and Rakita, S. (2015). Albumins Characterization in Relation to Rheological Properties and Enzymatic Activity of Wheat Flour Dough. *Journal of Agricultural Science and Technology,* Vol. 17: 805-816.
29. **Osipova, S. V.**, Permyakova, M. D., and Permyakov, A. V. (2012). Role of Non-prolamin Proteins and Low Molecular Weight Redox Agents in Protein Folding and Polymerization in Wheat Grains and Influence on Baking Quality Parameters. *Journal of Agricultural and Food Chemistry,* 60: 12065−12073.
30. **Wieser, H.** (2007). Chemistry of gluten proteins. *Food Microbiology,* 24, 115-119.
31. **Biesiekierski, J. R.** (2017). What is gluten?. *Journal of Gastroenterology and Hepatology, 32,* 78-81.
32. **Schalk, K.**, Lexhaller, B., Koehler, P., Scherf, K.A.(2017). Isolation and characterization of gluten protein types from wheat, rye, barley and oats for use as reference materials. *PLoS One*, 12(2): e0172819.
33. **Ozuna, C. V.** & Barro, F. (2018). Characterization of gluten proteins and celiac disease-related immunogenic epitopes in the Triticeae: cereal domestication and breeding contributed to decrease the content of gliadins and gluten. *Molecular Breeding,* 38:22.
34. **Shewry, P. R.**, Halford, N. G., Belton, P. S. and Tatham, A. S. (2002). The structure and properties of gluten: an elastic protein from wheat grain. *Philosophical Transactions of the Royal Society B,* 357, 133–142.
35. **Tatham, A. S.**, Shewry, P. R. (2012). The S-poor prolamins of wheat, barley and rye: Revisited. *Journal of Cereal Science*, Vol. 55, 79-99.
36. **Tatham, A. S.**, Shewry, P. R. (2008). Allergens in wheat and related cereals. *Clinical and Experimental Allergy*, 38:1712–1726.
37. **Tosi, P.**, Parker, M., Gritsch, C. S., Carzaniga, R., Martin, B. and Shewry, P. R. (2009). Trafficking of storage proteins in developing grain of wheat. *Journal of Experimental Botany*, Vol. 60, No. 3, 979–991.
38. **Lamacchia, C.**, Camarca, A., Picascia, S., Di Luccia, A. and Gianfrani, C. (2014). Cereal-Based Gluten-Free Food: How to Reconcile Nutritional and Technological Properties of Wheat Proteins with Safety for Celiac Disease Patients. *Nutrients,* 6, 575-590.
39. Shewry, P. R. and Tatham, A. S. (1997). Disulphide Bonds in Wheat Gluten Proteins. *Journal of Cereal Science,* 25, 207–227.
40. Field, J.M., Shewry, P.R., Miflin, B.J. (1983). Solubilization and characterization of wheat gluten proteins; correlations between the amount of aggregated proteins and baking quality. *Journal of the Science of Food and Agriculture,* 34, 370–377.
41. Payne, P.I. (1987). Genetics of wheat storage and the effect of allelic variation on breadmaking quality. *Annual Review of Plant Biology,* 38, 141–153.
42. **Shewry, P.R.** (1999). The synthesis, processing, and deposition of gluten proteins in the developing wheat grain. *Cereal Foods World,* 44, 587–589.
43. Lindsay, M.P., Skerritt, J.H. (1999). The glutenin macropolymer of wheat flour doughs: Structure-function perspective. *Trends in Food Science & Technology,* 10, 247–253.
44. Shewry, P.R., Popineau, Y., Lafiandra, D., Belton, P. (2001). Wheat glutenin subunits and dough elasticity: Findings of the EUROWHEAT project. *Trends in Food Science & Technology,* 11, 433–441.
45. Wieser, H., Bushuk, W., Mac Ritchie, F. (2006). The Polymeric Glutenins. In Gliadin and Glutenin: The Unique Balance of Wheat Quality. St. Paul American Association of Cereal Chemistry; Wrigley, C., Bekes, F., Bushuk, W., Eds.; American. Association of Cereal Chemists: St. Paul MN, USA, 213–240.
46. Lagrain, B., Brunnbauer, M., Rombouts, I., Koehler, P. (2013). Identification of Intact High Molecular Weight Glutenin Subunits from the Wheat Proteome Using Combined Liquid Chromatography-Electrospray Ionization Mass Spectrometry. *PLoS ONE,* 8(3): e58682.
47. Tilley, K. A., Benjamin, R.E., Bagorogoza, K.E., Okot-Kotber, B. M., Prakash, O., and Kwen, H. (2001). Tyrosine Cross-Links: Molecular Basis of Gluten Structure and Function. *Journal of Agricultural and Food Chemistry*, 49, 2627−2632.
48. **Urade, R**. & Sato, N. & Sugiyama, M. (2018). Gliadins from wheat grain: an overview, from primary structure to nanostructures of aggregates. *Biophysical Reviews,* 10:435–443.
49. Kasarda, D.D. (1980). Structure and properties of α-gliadin. *Annals of Agricultural Sciences,* 29:151–173.
50. Cole, E.W., Kasarda, D.D., Lafiandra, D. (1984) The conformational structure of α-gliadin intrinsic viscosities under conditions approaching the native state and under denaturing conditions. *Biochimica et Biophysica Acta,* 787:244–2.

51. Ukai, T., Matsumura, Y., Urade, R. (2008) Disaggregation and reaggregation of gluten proteins by sodium chloride. *Journal of Agricultural and Food Chemistry*, 56:1122– 1130.
52. Shewry, P. R., Lafiandra, D., Tamas, L., and Bekes, F. (2006). Genetic manipulation of gluten structure and function. In: C. Wrigley, F. Bekes, and W. Bushuk (Eds.), *Gliadin and gluten in the unique balance of wheat quality.* St Paul, US: American Association of Cereal Chemistry.
53. Lamacchia, C., Landriscina, L., D'Agnello, P. (2016). Changes in wheat kernels proteins induced by microwave treatment. *Food Chemistry,* 197, 634-640.
54. D'Ovidio, R., Marchitelli, C., Ercoli Cardelli, L., and Porceddu, E. (1999). Sequence similarity between allelic Glu-B3 genes related to quality properties of durum wheat. *Theoretical and Applied Genetics*, 98:455-461.
55. Shewry, P.R. (2000) Seed Proteins. In *Seed Technology and Its Biological Basis*; Black, M., Bewley, J.D., Eds.; Sheffield Academic Press: Sheffield, UK; 42–84.
56. Combs, G.F. (2001). Selenium in global food systems. *British Journal of Nutrition*, 85, 517–547.
57. Cao, L., Liu, X., Qian, T., Sun, G., Guo, Y., Chang, F., Zhou, S., Sun, X. (2011). Antitumor and immunomodulatory activity of arabinoxylans: a major constituent of wheat bran. *International Journal of Biological Macromolecules*, 48(1):160-4.
58. Balk, J., Connorton, J.M., Wan, Y., Lovegrove, A., Moore, K.L., Uauy, C., Sharp, P.A., Shewry, P.R. (2019). Improving wheat as a source of iron and zinc for global nutrition. *Nutrition Bulletin*, 44(1):53-59.
59. Tosi, P., Gritsch, C.S., He, J., Shewry, P.R. (2011). Distribution of gluten proteins in bread wheat (Triticum aestivum) grain. *Annals of Botany,* 108(1):23-35.
60. Lamacchia, C., Shewry, P.R., Di Fonzo, N. et al. (2001). Endosperm specific activity of a storage protein gene promoter in transgenic wheat seed. *Journal of Experimental Botany,* 52: 243–250.
61. Ludvigsson, J. F., Leffler, D.A., Bai, J., Biagi, F., Fasano, A., Green, P. H.R., Hadjivassiliou, M., M.D., Kaukinen, K., Ciaran, K., Leonard, J.N., Lundin, K. E., Murray, J. A., Sanders, D. S., Walker, M. M., Zingone, F., and Ciacci, C. (2013). The Oslo definitions for coeliac disease and related terms. *Gut*, 62: 43–52.
62. Ludvigsson, J.F., Ciacci, C., Green, P.H., Kaukinen, K., Korponay-Szabo, I.R., Kurppa, K., Murray, J.A., Lundin, KEA, Maki, M.J., Popp, A., Reilly, N.R., Rodriguez-Herrera, A., Sanders, D.S., Schuppan, D., Sleet, S., Taavela, J., Voorhees, K., Walker, M.M., Leffler, D.A. (2018). Outcome measures in coeliac disease trials: the Tampere recommendations. *Gut*, 67:1410-1424.
63. Nanayakkara, M., Lania, G., Maglio, M., Kosova, R., Sarno, M., Gaito, A., Discepolo, V., Troncone, R., Auricchio, S., Auricchio, R., Barone, M.V. (2013). Enterocyte proliferation and signaling are constitutively altered in celiac disease. *PLoS One*, 8(10):e76006.
64. De Re, V., Magris, R., Cannizzaro, R. (2017). New Insights into the Pathogenesis of Celiac Disease. *Frontiers in Medicine*, 31; 4:137.
65. Clemente, M.G., De Virgiliis, S., Kang, J. S., Macatagney, R., Musu, M. P., DiPierro, M. R., Drago, S., Congia, M., Fasano, A. (2003). Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. *Gut*, 52:218–223.
66. Barone, M.V., Troncone, R., Auricchio, S. (2014). Gliadin Peptides as Triggers of the Proliferative and Stress/Innate Immune Response of the Celiac Small Intestinal Mucosa. *International Journal of Molecular Sciences*, 7;15(11):20518-37.
67. Mazzarella, G., Stefanile, R., Camarca, A., Giliberti, P., Cosentini, E., Marano, C., Iaquinto, G., Giardullo, N., Auricchio, S., Sette, A., Troncone, R., Gianfrani, C. (2008). Gliadin Activates HLA Class I-Restricted CD8+ T Cells in Celiac Disease Intestinal Mucosa and Induces the Enterocyte Apoptosis. *Gastroenterology*, 134(4): 1017–1027.
68. Schuppan, D., Junker, Y., Barisani, D. (2009). Celiac disease: from pathogenesis to novel therapies. *Gastroenterology*, 137(6):1912-33.
69. Meresse, B., Malamut, G., Cerf-Bensussan, N. (2012). Celiac disease: an immunological jigsaw. *Immunity,* ;36(6):907-19.
70. Lundin, K.E., Sollid, L.M. (2014). Advances in coeliac disease. *Current Opinion in Gastroenterology*, 30(2):154-62.
71. Green, P. H.R., Lebwohl, B., Greywoode, R. (2015). Celiac disease. *Clinical reviews in allergy and immunology*, 135:1099-106.
72. Hardy, M.Y., Tye-Din, J.A. (2016). Coeliac disease: a unique model for investigating broken tolerance in autoimmunity. *Clinical & Translational Immunology*, 5(11):e112.
73. Lebwohl, B., Ludvigsson, J.F., Green, P.H.(2014). The unfolding story of celiac disease risk factors. *Clinical Gastroenterology and Hepatology*, 12(4):632-5.

74. Kim, S.M., Mayassi, T., Jabri, B. (2015). Innate immunity: actuating the gears of celiac disease pathogenesis. *Best Practice & Research: Clinical Gastroenterology*, 29(3):425-35.
75. Aliberti, J. (2016). Immunity and Tolerance Induced by Intestinal Mucosal Dendritic Cells. *Mediators of Inflammation*, 2016:3104727.
76. Moheb-Alian, A., Forouzesh, F., Rostami-Nejad, M., Rostami, K. (2016). Mesenchymal stem cells as potential therapeutic approaches in celiac disease. *Gastroenterology and Hepatology from Bed to Bench*, 9 (Suppl1): S1-S7.
77. Felli, C., Baldassarre, A., Masotti, A. (2017). Intestinal and Circulating MicroRNAs in Coeliac Disease. *International Journal of Molecular Sciences*, 18(9).
78. Miner-Williams, W.M., Moughan, P.J. (2016). Intestinal barrier dysfunction: implications for chronic inflammatory conditions of the bowel. *Nutrition Research Reviews*, 29(1):40-59.
79. Vancamelbeke, M., Vermeire, S. (2017). The intestinal barrier: a fundamental role in health and disease. *Expert Review of Gastroenterology & Hepatology*, 11(9):821-834.
80. Fasano, A. (2011). Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer. *Physiological Reviews*, 91(1):151-75.
81. Lebwohl, B., Ludvigsson, J.F., Green, P.H. (2015). Celiac disease and non-celiac gluten sensitivity. *British Medical Journal*, 351:4347.
82. Caio, G., Volta, U., Sapone, A., Leffler, D.A., De Giorgio, R., Catassi, C., Fasano, A. (2019). Celiac disease: a comprehensive current review. *BMC Medical Education*, 17(1):142.
83. Ludvigsson, J.F., Murray, J.A. (2019). Epidemiology of Celiac Disease. *Gastroenterology Clinics of North America*, 48(1):1-18.
84. Makharia, G.K., Catassi, C. (2019). Celiac Disease in Asia. Gastroenterology Clinics of North America, 48(1):101-113.
85. Ashtari, S., Pourhoseingholi, M.A., Rostami, K., Aghdaei, H.A. et al. (2019). Prevalence of gluten-related disorders in Asia-Pacific region: a systematic review. *Journal of Gastrointestinal and Liver Diseases,* 28(1):95-105.
86. Jansson-Knodell, C.L., Hujoel, I.A., West, C.P., Taneja, V., Prokop, L.J., Rubio-Tapia, A., Murray, J.A. (2018). Sex Difference in Celiac Disease in Undiagnosed Populations: A Systematic Review and Meta-analysis. *Clinical Gastroenterology and Hepatology*, S1542-3565(18)31256-4.
87. Barker, J. M., and Liu, E. (2008). Celiac Disease: Pathophysiology, Clinical Manifestations and Associated Autoimmune Conditions. *Advances in Pediatrics*, 55: 349–365.
88. **Singh, P.**, Arora, A., Strand, T.A., Leffler, D.A., Catassi, C., Green, P.H., Kelly, C.P., Ahuja, V., Makharia, G.K. (2018). Global Prevalence of Celiac Disease: Systematic Review and Meta-analysis. *Clinical Gastroenterology and Hepatology*, 16(6):823-836.
89. Mäki, M., Mustalahti, K., Kokkonen, J., Kulmala, P., Haapalahti, M., Karttunen, T., Ilonen, J., Laurila, K., Dahlbom, I., Hansson, T., Höpfl, P., Knip, M. (2003). Prevalence of Celiac disease among children in Finland. *The New England Journal of Medicine*, 348(25):2517-24.
90. Wacklin, P., Laurikka, P., Lindfors, K., Collin, P., Salmi, T., Lähdeaho, M.L., Saavalainen, P., Mäki, M., Mättö, J., Kurppa, K., Kaukinen, K. (2014). Altered duodenal microbiota composition in celiac disease patients suffering from persistent symptoms on a long-term gluten-free diet. *The American Journal of Gastroenterology*, 109(12):1933-41.
91. Papadopoulos, G.K., Wijmenga, C., Koning, F. (2001). Interplay between genetics and the environment in the development of celiac disease: perspectives for a healthy life. *Journal of Clinical Investigation*, 108(9):1261-6.
92. Marasco, G., Di Biase, A.R., Schiumerini, R., Eusebi, L.H., Iughetti, L., Ravaioli, F., Scaioli, E., Colecchia, A., Festi, D. (2016). Gut Microbiota and Celiac Disease. *Digestive Diseases and Sciences*, 61(6):1461-72.
93. Guandalini, S., Assiri, A. (2014). Celiac disease: a review. *JAMA Pediatrics*, 168:272–278.
94. Abadie, V., Sollid, L.M., Barreiro, L.B., Jabri, B. (2011). Integration of genetic and immunological insights into a model of celiac disease pathogenesis. *Annual Review of Immunology*, 29:493–525.
95. Maiuri, L., Ciacci, C., Ricciardelli, I., et al. (2003). Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. *Lancet*, 362:30–37.
96. Sapone, A., Bai, J.C., Ciacci, C., Dolinsek, J., Green, P.H., Hadjivassiliou, M., Kaukinen, K., Rostami, K., Sanders, D.S., Schumann, M., Ullrich, R., Villalta, D., Volta, U., Catassi, C., Fasano, A. (2012). Spectrum of gluten-related disorders: consensus on new nomenclature and classification. *BMC Medicine*, 7; 10:13.

97. Stoven, S., Murray, J.A., Marietta, E.V. (2013). Latest In vitro and in vivo models of celiac disease. *Expert Opinion on Drug Discovery*, 8(4):445-57.
98. Jabri, B., Sollid, L.M. (2017). T Cells in Celiac Disease. *The Journal of Immunology*, 198(8):3005-3014.
99. Villanacci, V., Corazza, G.R.: Coeliac disease. (2005). *Journal of Clinical Pathology*, 58:573-574.
100. Pelaseyed, T., Bergström, J.H., Gustafsson, J.K., et al. (2014). The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunological Reviews*, 260(1):8–20.
101. Jakobsson, H.E., Rodríguez-Piñeiro, A.M., Schütte, A, et al. (2015). The composition of the gut microbiota shapes the colon mucus barrier. *EMBO Reports*,16(2):164–177.
102. Iversen, R., Roy, B., Stamnaes, J., Høydahl, L.S., Hnida, K., Neumann, R.S., Korponay-Szabó, I.R., Lundin, K.E.A, Sollid, L.M. (2019). Efficient T cell-B cell collaboration guides autoantibody epitope bias and onset of celiac disease. *Proceedings of the National Academy of Sciences*, 116(30):15134-15139.
103. Hossein-Nataj, H., Masjedi, M., Emami, M.H., Mokhtari, M., Alsahebfosoul, F. (2019). Cell Density Counts of the Intestinal Intraepithelial Lymphocytes in the Celiac Patients. *Iranian Journal of Immunology*, 16(2):117-126.
104. Rescigno, M., Urbano, M., Valzasina, B., et al. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Immunology*, 2(4):361–367.
105. Hall, J. A., Grainger, J. R., Spencer, S. P., Belkaid, Y. (2011). The role of retinoic acid in tolerance and immunity. *Immunity*, 35(1):13–22.
106. Baba, N., Samson, S., Bourdet-Sicard, R. L., Rubio, M., Sarfati, M. (2008). Commensal bacteria trigger a full dendritic cell maturation program that promotes the expansion of non-Tr1 suppressor T cells. *Journal of Leukocyte Biology*, 84(2):468–476.
107. Coombes, J. L., Siddiqui, K. R. R., Arancibia-Carcamo, C. V., et al. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-β− and retinoic acid-dependent mechanism. *The Journal of Experimental Medicine*, 204(8):1757–1764.
108. Yu, Q.H., Yang, Q. (2009). Diversity of tight junctions (TJs) between gastrointestinal epithelial cells and their function in maintaining the mucosal barrier. *Cell Biology International*, 33(1):78-82.
109. Plenge, R.M. (2010). Unlocking the pathogenesis of celiac disease. *Nature Genetics*, 42(4):281-2.
110. Camarca, A., Anderson, R.P., Mamone, G., Fierro, O., Facchiano, A., Costantini, S., Zanzi, D., Sidney, J., Auricchio, S., Sette, A., Troncone, R., Gianfrani, C. (2009). Intestinal T cell responses to gluten peptides are largely heterogeneous: implications for a peptide-based therapy in celiac disease. *The Journal of Immunology,* 182: 4158–4166.
111. Thomas, K.E., Fasano, A., Vogel, SN. (2006). Gliadin stimulation of murine macrophage inflammatory gene expression and intestinal permeability are MyD88-dependent: role of the innate immune response in Celiac disease. *The Journal of Immunology*, 176: 2512–2521.
112. Turner, J.R. (2009). Intestinal mucosal barrier function in health and disease. *Nature Reviews Immunology*, 9: 799–809.
113. Frossi, B., De Carli, M., Calabrò, A. (2019). Coeliac Disease and Mast Cells. *International Journal of Molecular Sciences*, 20(14).
114. Cicerone, C., Nenna, R., Pontone, S. (2015). Th17, intestinal microbiota and the abnormal immune response in the pathogenesis of coeliac disease. *Gastroenterology and Hepatology from Bed to Bench*, 117–122.
115. Patidar, M., Yadav, N. Dalai, S.K. (2016). Interleukin 15: A key cytokine for immunotherapy. *Cytokine & Growth Factor Reviews*, 31, 49–59.
116. Ebert, E.C. (1998). Interleukin 15 is a potent stimulant of intraepithelial lymphocytes. *Gastroenterology*, 115, 1439–1445.
117. Fehniger, T.A., Caligiuri, M.A. (2001). Interleukin 15: Biology and relevance to human disease. *Blood*, 97, 14–32.
118. Cua, D.J., Tato, C.M. (2010). Innate IL-17-producing cells: the sentinels of the immune system. *Nature Reviews Immunology*, 10:479-89.
119. Blaschitz, C., Raffatellu, M. (2010). Th17 cytokines and the gut mucosal barrier. *Journal of Clinical Immunology*, 30:196-203.
120. Proctor, L.M. (2011). The human microbiome project in 2011 and beyond. *Cell Host Microbe*, 10:287-91.
121. Decker, E., Engelmann, G., Findeisen, A., et al. (2010). Cesarean delivery is associated with celiac disease but not inflammatory bowel disease in children. *Pediatrics*, 125(6):1433–1440.

122. Namatovu, F., Olsson, C., Lindkvist, M., et al. (2016). Maternal and perinatal conditions and the risk of developing celiac disease during childhood. *BMC Pediatrics*, 16(1):77.
123. Dydensborg Sander, S., Hansen, A.V., Størdal, K., Andersen, A.N., Murray, J.A., Husby, S. (2018). Mode of delivery is not associated with celiac disease. *Journal of Clinical Epidemiology*, 10:323-332.
124. Mårild, K., Ye, W., Lebwohl, B., Green, P. H.R., et al. (2013). Antibiotic exposure and the development of coeliac disease: a nationwide case–control study. *BMC Gastroenterology*, 13: 109.
125.Van den Broeck, H.C., de Jong, H.C., Salentijn, E.M., et al. (2010). Presence of celiac disease epitopes in modern and old hexaploid wheat varieties: wheat breeding may have contributed to increased prevalence of celiac disease. *Theoretical and Applied Genetics*, 121:1527–39.
126. Bach, J.F. (2018). The hygiene hypothesis in autoimmunity: the role of pathogens and commensals. *Nature Reviews Immunology*, 18:105–20.
127. Mu, Q., Kirby, J., Reilly, C.M., Luo, X.M. (2017). Leaky Gut As a Danger Signal for Autoimmune Diseases. *Frontiers in Immunology*, 23; 8:598.
128. Peterson, L.W., Artis, D. (2014). Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nature Reviews Immunology*, 14(3):141–53.10.
129. Salentijn, E.M., Mitea, D.C., Goryunova, S.V., van der Meer, I.M., Padioleau, I., Gilissen, L.J., Koning, F., Smulders, M.J. (2012). Celiac disease T-cell epitopes from gamma-gliadins: immunoreactivity depends on the genome of origin, transcript frequency, and flanking protein variation. *BMC Genomics*, 13:277.
130. Agarwal, S., Kovilam, O., Zach, T.L., Agrawal, D.K. (2016). Immunopathogenesis and therapeutic approches in pediatric celiac disease. *Expert Rev. Clinical Immunology*, 12: 857-69.
131. Verdu, E.F., Galipeau, H.J, Jabri, B. (2015). Novel player in coeliac disease pathogenesis: role of the gut microbiota. Nature Rev. *Gastroenterology Hepatology*, 12: 497-506.
132. Tye-Din, J.A., Galipeau, H.J., Agardh, D. (2018). Celiac Disease: A Review of Current Concepts in Pathogenesis, Prevention, and Novel Therapies. *Frontiers in Pediatrics*, 21; 6:350.
133. Abadie, V., Discepolo, V., Jabri, B. (2012). Intraepithelial lymphocytes in celiac disease immunopathology. *Seminars In Immunopathology*, 34(4):551-66.
134. Abadie, V., Jabri, B. (2014). IL-15: a central regulator of celiac disease immunopathology. *Immunological Reviews*, 260(1):221-34.
135. Bouziat, R., Hinterleitner, R., Brown, J.J., Stencel-Baerenwald, J.E., Ikizler, M., Mayassi, T., Meisel, M., et al. (2017). Reovirus infection triggers inflammatory responses to dietary antigens and development of celiac disease. *Science*, 356: 44-50.
136. Kupfer, S. S., Jabri, B. (2012). Celiac Disease Pathophysiology. *Gastrointestinal Endoscopy Clinics of North America*, 22(4):639-60.
137. Lionetti, E., Catassi, C. (2015). The Role of Environmental Factors in the Development of Celiac Disease: What Is New? *Diseases*, 3, 282-293.
138. Lopetuso, L.R., Scaldaferri, F., Bruno, G., Petito, V., Franceschi, F., Gasbarrini, A. (2015). The therapeutic management of gut barrier leaking: the emerging role for mucosal barrier protectors. *European Review for Medical and Pharmacological Sciences*, 19(6):1068-76.
139. Leonard, M.M., Sapone, A., Catassi, C., Fasano, A. (2017). Celiac Disease and Non-celiac Gluten Sensitivity: A Review. *JAMA*, 318(7):647-656.
140. Buckley, A., Turner, J.R. (2018). Cell Biology of Tight Junction Barrier Regulation and Mucosal Disease. *Cold Spring Harb Perspect Biol.* 2;10(1).
141. Shen, L., Weber, C.R., Raleigh, D.R., Yu, D., Turner, J.R. (2011). Tight junction pore and leak pathways: a dynamic duo. *Annual Review of Physiology*, 73:283-309.
142. Jauregi-Miguel, A., Fernandez-Jimenez, N., Irastorza, I., Plaza-Izurieta, L., Vitoria, J.C., Bilbao, J.R. (2014). *Journal of Pediatric Gastroenterology and Nutrition*, 58(6):762-7.
143. Jung, C., Hugot, J.P., Barreau, F. (2010). Peyer's Patches: The Immune Sensors of the Intestine. *International Journal of Inflammation*, 2010:823710.
144. Johansson, M.E., Hansson, G.C. (2016). Immunological aspects of intestinal mucus and mucins. *Nature Reviews Immunology*, 16(10):639-49.
145. Shan, M., Gentile, M., Yeiser, J.R., Walland, A.C., et al. (2013). Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. *Science*, 342(6157):447-53.
146. Rao, R.K., Samak, G. (2013). Protection and Restitution of Gut Barrier by Probiotics: Nutritional and Clinical Implications. *Current Nutrition & Food Science*, 9(2):99-107.
147. Lavelle, A., Hill, C. (2019). Gut Microbiome in Health and Disease: Emerging Diagnostic Opportunities. *Gastroenterology Clinics of North America*, 48(2):221-235.

148. Shreiner, A.B., Kao, J.Y., Young, V.B. (2015). The gut microbiome in health and in disease. *Current Opinion in Gastroenterology*, 31(1):69-75.
149. Round, J.L and Mazmania, S.K. (2009). The gut microbiome shapes intestinal immune responses during health and disease. *Nature Reviews Immunology*, 9(5): 313–323.
150. Lin, L., Zhang, J. (2017). Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. *BMC Immunology*, 6;18(1):2.
151. Blacher, E., Levy, M., Tatirovsky, E., Elinav, E. (2017). Microbiome-Modulated Metabolites at the Interface of Host Immunity. *The Journal of Immunology*, 198(2):572-580.
152. Sharma, R., Young, C., Neu, J. (2010). Molecular modulation of intestinal epithelial barrier: contribution of microbiota. *Journal of Biomedicine and Biotechnology*, 2010:305879.
153. Rescigno, M. (2008). Intestinal epithelial cells control dendritic cell function. *Journal of Pediatric Gastroenterology and Nutrition*, 46 Suppl 1: E17-9.
154. Rescigno, M. (2017). The microbiota revolution: Excitement and caution. *European Journal of Immunology*, 47(9):1406-1413.
155. Kelsall, B.L, Rescigno, M. (2004). Mucosal dendritic cells in immunity and inflammation. *Nature Immunology*, 5(11):1091-5.
156. Krishnan, S., Alden, N., Lee, K. (2015). Pathways and functions of gut microbiota metabolism impacting host physiology. *Current Opinion in Biotechnology*, 36:137-45.
157. Rimoldi, M., Chieppa, M., Salucci, V., Avogadri, F., Sonzogni, A., Sampietro, G.M., Nespoli, A., Viale, G., Allavena, P., Rescigno, M. (2005). Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nature Immunology*, 6(5):507-14.
158. Rescigno, M., Lopatin, U., Chieppa, M. (2008). Interactions among dendritic cells, macrophages, and epithelial cells in the gut: implications for immune tolerance. *Current Opinion in Immunology*, 20(6):669-75.
159. Cukrowska, B., Sowińska, A., Bierła, J.B., Czarnowska, E., Rybak, A., Grzybowska-Chlebowczyk, U. (2017). Intestinal epithelium, intraepithelial lymphocytes and the gut microbiota - Key players in the pathogenesis of celiac disease. *World Journal of Gastroenterology*, 23(42):7505-7518.
160. Girbovan, A., Sur, G., Samasca, G., Lupan, I. (2017). Dysbiosis a risk factor for celiac disease. *Medical Microbiology and Immunology*, 206(2):83-91.
161. Krishnareddy, S. (2019). The Microbiome in Celiac Disease. *Gastroenterology Clinics of North America*, 48(1):115-126.
162. Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G.A.D., Gasbarrini, A., Mele, M.C. (2019). What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms*, 7(1). pii: E14.
163. Wu, T.S., Lee, J.M., Lai, Y.G., Hsu, J.C., Tsai, C.Y., Lee, Y.H., et al. (2002). Reduced expression of Bcl-2 in CD8+ T cells deficient in the IL-15 receptor alpha-chain. *The Journal of Immunology*, 168:705–12.
164. Benahmed, M., Meresse, B., Arnulf, B., Barbe, U., Mention, J.J., Verkarre, V., et al. (2007). Inhibition of TGF-beta signaling by IL-15: a new role for IL-15 in the loss of immune homeostasis in celiac disease. *Gastroenterology*, 132:994–1008.
165. Meisel, M., Mayassi, T., Fehlner-Peach, H., et al. (2017). Interleukin-15 promotes intestinal dysbiosis with butyrate deficiency associated with increased susceptibility to colitis.*The ISME Journal*; 11(1): 15–30.
166. Ihara, S., Hirata, Y., Serizawa, T., Suzuki, N., et al. (2016). TGF-β signaling in dendritic cells governs colonic homeostasis by controlling epithelial differentiation and the luminal microbiota. *The Journal of Immunology*, 196 (11) 4603-4613.
167. Hansson, T., Dannaeus, A., Klareskog, L. (1999). Cytokine-producing cells in peripheral blood of children with coeliac disease secrete cytokines with a type 1 profile. *Clinical & Experimental Immunology*, 116(2):246-50.
168. Girard-Madoux, M., Ober-Blöbaum, J., Costes, L., Kel, J.M., et al. **(**2016). IL-10 control of CD11c+ myeloid cells is essential to maintain immune homeostasis in the small and large intestine. *Oncotarget*, 7(22):32015-30.
169. Iacomino, G., Marano, A., Stillitano, I., Aufiero, V.R., et al. (2016). Celiac disease: role of intestinal compartments in the mucosal immune response. *Molecular and Cellular Biochemistry*, 411(1-2):341-9.
170. Vorobjova, T., Tagoma, A., Oras, A., Alnek, K., Kisand, K. (2019). Celiac Disease in Children, Particularly with Accompanying Type 1 Diabetes, Is Characterized by Substantial Changes in the Blood Cytokine Balance, Which May Reflect Inflammatory Processes in the Small Intestinal Mucosa. *Journal of Immunology Research*, 2019:6179243.

171. Wierdsma, N.J.; van Bokhorst-de van der Schueren, M.A.; Berkenpas, M.; Mulder, C.J.; van Bodegraven, A.A. (2013). Vitamin and mineral deficiencies are highly prevalent in newly diagnosed celiac disease patients. *Nutrients,* 5, 3975–3992.
172. Rostami, K., Bold, J., Parr, A., Johnson, M.W. (2017). Gluten-Free Diet Indications, Safety, Quality, Labels, and Challenges. *Nutrients,* 8;9(8).
173. Schreiner, P., Yilmaz, B., Rossel, J.B., Franc, Y., Misselwitz, B., Scharl, M., Zeitz, J. et al. (2019). Vegetarian or gluten-free diets in patients with inflammatory bowel disease are associated with lower psychological well-being and a different gut microbiota, but no beneficial effects on the course of the disease. *United European Gastroenterology Journal*, 7(6):767-781.
174. Ojetti, V., Nucera, G., Migneco, A., Gabrielli, M., Lauritano, C., Danese, S., Zocco, M.A., Nista, E.C., Cammarota, G., De Lorenzo, A., Gasbarrini, G., Gasbarrini, A.(2005). High prevalence of celiac disease in patients with lactose intolerance. *Digestion*, 71(2):106-10.
175. Bascuñán, K.A., Vespa, M.C., Araya, M. (2017). Celiac disease: understanding the gluten-free diet. *European Journal of Nutrition*, 56(2):449-459.
176. Itzlinger, A., Branchi, F., Elli, L., Schumann, M. (2018). Gluten-Free Diet in Celiac Disease-Forever and for All? *Nutrients*, 10(11).
177. Kasarda, D.D. (2013). Can an increase in celiac disease be attributed to an increase in the gluten content of wheat as a consequence of wheat breeding? *Journal of Agricultural and Food Chemistry*, 13;61(6):1155-9.
178. **Gambus, H.**; Gambus, F.; Sabat, R.(2002). The research on quality improvement of gluten-free bread by amaranthus flour addition. *Zywnosc*, 9, 99–112
179. Taylor, J.R.N.; Parker, M.L. (2002). Quinoa. In Pseudocereals and Less Common Cereals, Grain Properties and Utilization Potential; Springer: Verlag, Berlin, 93–122.
180. Mukherjee, R., Kelly, C.P., Schuppan, D. (2012). Nondietary therapies for celiac disease. *Gastrointestinal Endoscopy Clinics of North America,* 22(4):811–831.
181. Mazzarella, G., Salvati, V.M.; Iaquinto, G.; Stefanile, R.; Capobianco, F.; Luongo, D.; Bergamo, P.; Maurano, F.; Giardullo, N.; Malamisura, B.; et al. (2012). Reintroduction of gluten following flour transdamidation in adult celiac patients: A randomized, controlled clinical study. *Clinical and Developmental Immunology*, 329150.
182. Rai, S., Kaur, A., Chopra, C.S. (2018). Gluten-Free Products for Celiac Susceptible People. *Frontiers in Nutrition*, 17; 5:116.
183. Sánchez-León, S., Gil-Humanes, J., Ozuna, C.V., Giménez, M.J., Sousa, C., Voytas, D.F., Barro, F. (2018). Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. Plant Biotechnology Journal, 16(4):902-910.
184. García-Molina, M D., Giménez, M.J., Sánchez-León, S., Barro, F. (2019). Gluten Free Wheat: Are We There? *Nutrients*, 26;11(3).
185. **Lamacchia, C.**, Di Luccia, A., and Gianfrani, C. (2013). Method for detoxification of gluten protein from grains of cereals. Patent Cooperation Treaty; PCT/IB2013/000797.
186. Lamacchia, C., Di Luccia, A., & Gianfrani, C. (2015). Metodo per la detossificazione delle proteine del glutine dalle granaglie dei cereali. Italian patent n°. 0001414717. April 26th 2015.
187. Lamacchia, C., Baiano, A., Lamparelli, S., La Notte, E., Di Luccia, A. (2010). Changes in durum wheat kernel and pasta proteins induced by toasting and drying processes. *Food Chemestry*, 118, 191-198.
188. Lamacchia, C., Landriscina, L., & D'Agnello, P. (2016). Changes in wheat kernel proteins induced by microwave treatment. *Food Chemistry*, 197, 634–640.
189. Landriscina, L., D'Agnello, P., Bevilacqua, A., Corbo, M. R., Sinigaglia, M., & Lamacchia, C. (2017). Impact of gluten-friendly™ technology on wheat kernel endosperm and gluten protein structure in seeds by light and electron microscopy. *Food Chemistry*, 221, 1258–1268.
190. Bevilacqua, A., Costabile, A., Bergillos-Meca, T., Gonzalez, I., Landriscina, L., Ciuffreda, E., D'Agnello, P., Corbo, M.R., Sinigaglia, M., Lamacchia, C. (2016). Impact of Gluten-Friendly Bread on the Metabolism and Function of In Vitro Gut Microbiota in Healthy Human and Coeliac Subjects. *PLoS One*, 1(9): e0162770.
191. Costabile, A., Bergillos-Meca, T., Landriscina, L., Bevilacqua, A., Gonzalez-Salvador, I., Corbo, M.R., Petruzzi, L., Sinigaglia, M., Lamacchia, C. (2017). An In Vitro Fermentation Study on the Effects of Gluten FriendlyTM Bread on Microbiota and Short Chain Fatty Acids of Fecal Samples from Healthy and Celiac Subjects. *Frontiers in Microbiology*, 8:1722.
192. Lamacchia, C. (2016). Method for detoxification of gluten protein from grains of cereals and related medical uses; Italian priority patent n°:102015000084813, Patent Cooperation Treaty PCT/EP2016/0815899.

193. Bowman, K.A., Broussard, E.K., Surawicz, C.M. (2015). Fecal microbiota transplantation: current clinical efficacy and future prospects. Clin Exp Gastroenterol. 23;8:285-91. doi: 10.2147/CEG.S61305.

Chapter 2

Temperature-treated gluten proteins in Gluten-Friendly™ bread increase mucus production and gut-barrier function in human intestinal goblet cells

Temperature-treated gluten proteins in Gluten-Friendly™ bread increase mucus production and gut-barrier function in human intestinal goblet cells

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Carmela Lamacchia [a, *], Daniela Musaico [a,b], Mary E. Henderson [b], Triana Bergillos-Meca [b], Morgane Roul [b], Loretta Landriscina [a], Ivana Decina [a], Giulia Corona [b,1], Adele Costabile [b,1]

[a] *Department of the Science of Agriculture, Food and Environment, University of Foggia, Foggia, Italy*
[b] *Life Sciences Department, Whitelands College, University of Roehampton, London, United Kingdom*

[*] **Corresponding author.**
E-mail address: carmela.lamacchia@unifg.it (C. Lamacchia).
[1]These authors share co-senior authorship.

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Abstract

The effects of a control bread (CB) and a Gluten Friendly™ bread (GFB) on intestinal epithelium mucus production and barrier function in healthy human mucus-secreting goblet cells HT-29-16E were investigated. Mucus production in cells exposed to digested breads (GFB and CB) was preliminarily investigated using staining techniques, Periodic Acid-Schiff (PAS) and Alcian blue (AB), and MUC2 and MUC3 were also quantified by ELISA assay. The barrier function of the cell monolayer was evaluated by trans-epithelial electrical resistance (TEER) measurements. GFB increased the secretion of mucins, expressed as the level of PAS and AB staining in comparison with the control. MUC3 levels were not affected, whereas higher MUC2 concentrations ($P < 0.01$) were found on cells treated with GFB compared to the control. Additionally, significantly higher TEER values were observed after treatment with both CB and GFB in comparison with the control ($P < 0.01$), with GFB having a significantly higher effect than CB ($P < 0.01$).

Abbreviations: CD, coeliac disease; CB, control bread; GFB, Gluten-Friendly™ bread; GFD, Gluten-free diet; PAS, Periodic Acid-Schiff; AB, Alcian Blue; TEER, TransEpithelial Electrical Resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IBD, Inflammatory Bowel Diseases; IBS, (Irritable Bowel Syndrome); *nd, not determinable; ds, dry substance; W,dough strength; P, dough toughness; L, dough extensibility; A, water absorption; B, dough developing time; CD, dough stability*

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1. Introduction

The intestinal epithelial layer is a physical and biochemical barrier, formed by a monolayer of cells that define the boundary between tissues of the intestine and the external environment. Intestinal epithelial layer integrity confers protection against luminal antigens (Lundin & Sollid, 2014). The intestinal goblet cells are specialized secretory cells that are found in the epithelial layer. They are responsible for the production of mucins, which create a first layer of defence (Kim & Ho, 2010; Peterson & Artis, 2014) against intrusion of large particles and bacteria into the epithelial cell layer (Turner, 2009). The most external part, the non-adherent mucus layer, predominantly contains glycoproteins, including the gel-forming mucins MUC2, MUC5AC, MUC6 and MUC5B (Pelaseyed et al., 2014). The inner adherent layer contains transmembrane mucins, predominantly MUC3, MUC1, and MUC17 (Pelaseyed et al., 2014). Goblet cells secrete both the main gel-forming mucins, MUC2, and the transmembrane-bound mucins (Kim & Ho, 2010). These mucins are thought to additionally play an active role in regulating the porosity and permeability of the epithelial membrane (Peterson & Artis, 2014).

Below the mucous layers, the intestinal epithelial cells form a tight, continuous physical barrier, where adjacent epithelial cells are sealed together by tight junctions and adherens junctions (Schumann, Siegmund, Schulzke, & Fromm, 2016). These multi-protein tight junction complexes that join together the intestinal epithelial cells create a selectively permeable seal, marking out the divide between apical and basolateral membrane domains (Kim & Ho, 2010; Lee, 2015). By regulating the intestinal epithelial barrier function and its selective permeability, tight junction complexes determine the rate of flux of molecules across the epithelium (Wells et al., 2016).

A disturbed intestinal epithelial barrier function has been found in coeliac disease (CD) patients (Schulzke, Bentzel, Schulzke, Riecken, & Fromm, 1998), even after treatment, due to both reduced mucus secretion and increased intestinal cell permeability (Smecuol et al., 1997). This weakened gut barrier is more susceptible to pathogens (Schulzke et al., 1998) and potentially antigenic macromolecules, such as gluten (Alaedini & Green, 2005). Although disruption of intestinal barrier function in CD has been established, the precise nature of this dysfunction as both a cause and effect of CD is not clear (Schumann et al., 2016). Mechanistic evidence suggests that gluten increases intestinal cell permeability in sensitive individuals, and significantly reduces epithelial barrier function in CD patients compared to healthy controls. In patients adhering to a gluten-free diet (GFD), disruption of epithelial barrier function is partially reversed and intestinal permeability is improved, when compared to active CD patients not following a GFD (Fromm et al., 2009; Schumann et al., 2012). Currently, a lifelong GFD remains the only available treatment. However, achieving a lifestyle completely free of gluten is in reality often impossible, due to practical and social challenges, which

include the cross-contamination of foods (Hall, Rubin & Charnock, 2013; Thompson & Simpson, 2015). Furthermore, the effectiveness of a GFD varies among patients, whereas in non-responsive and refractory CD patients, GFD does not improve symptoms at all (Shannahan & Leffler, 2017). Moreover, the GFD is often low in vitamins and minerals, such as B vitamins, iron, calcium and zinc (Bascunan, Vespa & Araya, 2017; Olivares et al., 2015; Shannahan & Leffler, 2017), and is deficient in fiber (Hallert et al., 2002; Shepherd & Gibson, 2013). In recent years, a novel temperaturebased method called Gluten Friendly™ has been developed (Lamacchia, Landriscina & D'Agnello, 2016), where in vitro, the gluten becomes unrecognizable by gluten-specific antibodies, without removing gluten from the flour (Lamacchia et al., 2016). While the temperature-treated gluten loses its immunogenic properties *in vitro*, it does not lose its technological properties and viscoelasticity (its ability to create a matrix and form dough) (Lamacchia et al., 2016). Further *in vitro* studies conducted on wheat kernels treated with the Gluten Friendly™ method also link a reduced cross-reactivity of antibodies recognizing almost the entire range of gluten proteins, to conformational changes of gluten protein structure in the kernel (Landriscina et al., 2017). The effects of Gluten Friendly™ bread or flour on healthy subjects, celiac patients or individuals suffering from gluten-related disorders have not yet been investigated. However, it has been postulated *in vitro* that consuming GFB could contribute to maintaining the microbial balance in the gut (Bevilacqua et al., 2016; Costabile et al., 2017). Therefore, we aim to investigate the effects of the novel GFB, from temperature-treated wheat kernels, on mucus production and gut barrier function in cell culture experiments, using mucus-secreting intestinal goblet cells (HT29-16E) from healthy human models.

The reason why we selected bread instead of temperature-treated wheat kernels or flour is that we envisioned our study as a model which could explore a potential real-world application. In fact, in 2017 wheat was the second most common food crop produced in the world, after maize, as reported by the Food and Agriculture Organization (FAO). Bread is one of the most common ways in which wheat is processed for human consumption.

2. Materials and methods

2.1. Bread substrates and simulated in vitro gastrointestinal digestion

The wheat kernels were supplied by Casillo Group S.p.a (Corato, Italy) and the chemical/rheological of the flour (Table 1) and nutritional characteristics of the bread (Table 2) are provided.

Parameter	Amount
Moisture %	12.10
Protein %ds	11.50
Gluten %ds	7.50
Gluten index	80
Ash %ds	0.76
Falling number	403
Yellow index	Nd
Alveograph	W: 97–P/L: 0.73
Farinograph	A: 59.8–B: 2.1–CD: 2.5

Nd = not determinable; ds = drysubstance; W = dough strength; P = dough toughness; L = dough extensibility; A = water absorption; B = dough developing time; CD: dough stability.

Table 1. Chemical and rheological properties of soft wheat used in the study.

Energy	271.00/1134.00 (kcal/kj)	
Total fat	3.50 g	
	Saturated fat	0.85 g
	Polyunsaturated fat	1.39 g
	Monounsaturated	0.81 g
Total carbohydrate	50 g	
	Dietary fiber	2.70 g
	Sugars	0.83 g
Protein	9.6 g	
Cholesterol	0 mg	
Calcium	48 mg	
Sodium	530 mg	
Potassium	110 mg	
Iron	2.10 mg	
Magnesium	27 mg	
Thiamin (Vit. B1)	0.47 mg	
Riboflavin (Vit. B2)	0.15 mg	
Niacin (Vit. B3)	1.6 mg	

Table 2. Nutritional content of soft wheat bread per 100g.

The kernels underwent the Gluten Friendly™ temperature-based process as previously cited (Lamacchia et al., 2016; Landriscina et al., 2017). The caryopses were then milled into flour and baked into Gluten Friendly™ bread (GFB). A control bread (CB) was also baked using untreated

kernels. Both GFB and CB were prepared according to the same breadmaking process (100g wheat flour, 66mL water, 1.33g yeast, 1g salt) (Bevilacqua et al., 2016). GFB and CB were digested in vitro under appropriate conditions according to the procedures previously described (Maccaferri et al., 2012) in order to simulate mouth, stomach and small intestinal conditions. In particular, breads were homogenized with sterile distillated water in a stomacher. Then, digestive enzymes like α-amylase, pepsin, bile and pancreatine were added in due course under appropriate pH conditions in order to simulate mouth, stomach and small intestinal conditions, respectively. Once digested, the breads were then centrifuged at 2000g for 15min at room temperature to remove large particles and supernatants were filtered through a 0.2-μm sterile syringe filter in preparation for the cell culture experiments.

2.2. Cell lines

Human intestinal cells, HT29-19A (non-mucus-secreting) and HT29-16E (mucus-secreting) clones (Jarry, Merlin, Hopfer & Laboisse, 1994), were cultured using standard procedures with Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, UK) supplemented with foetal bovine serum (10%), 100× non-essential amino acids (1%), L-glutamine (1%) and penicillin/streptomycin (1%) (Sigma-Aldrich, UK) and maintained in a 5% CO_2 incubator at 37° C. All the assays were performed between the passages 12 and 20 (Davis, 2002).

2.3. Cytotoxic activity

The cytotoxic effect of CB and GFB breads was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (method (Schiller, Klainz,Mynett & Gescher, 1992). The HT29-16E and HT29-19A cells were seeded at a density of 2×105 cells/mL in 96-well plates. Monolayers for both cell lines were incubated for 5 days, until the subconfluence (at least 80%) was reached, and were fed on alternate days. In order to establish the optimal concentration to be used in the experiments, different concentrations of the digested breads (1%, 5%, 10%) were diluted in DMEM, and were then added to the cells and incubated for 24h at 37° C and 5% CO_2. An equivalent volume of DMEM was added to HT29-16E and HT29-19A control monolayers, which were also incubated for 24h at 37° C and 5% CO_2. MTT solution (0.5mg/mL) was then added and left for 3h at 37° C. The medium was then aspirated, and 100μL of DMSO was added to the cells. Colour development was measured at 570nm with a Multiskan EX spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). The cytotoxicity activity data were expressed as a percentage of viable cells, in relation to the control wells, which represent 100% viability.

2.4. Assessment of cell mucin production by Periodic acid Schiff (PAS) and Alcian blue staining

Mucus production was examined using both Periodic acid–Schiff (PAS) (Diebel, Liberati & Hall-Zimmerman, 2011; Schiller et al., 1992) and Alcian-blue (AB) (Steedman,1950) staining techniques. PAS is used to detect mucosubstances containing a high proportion of carbohydrate macromolecules, as well as polysaccharides, the basal laminae and the glycocalyx of cells. A positive PAS reaction indicates the presence of neutral mucins. A positive Alcian Blue reaction at pH 1.0 and 2.5 indicates the presence of acidic sulphated and acidic carboxylated mucins, respectively (Schumacher, Duku, Katoh, Jörns, & Krause, 2004; Tootian et al., 2013).

Transmembrane mucins MUC3, MUC12 and MUC17 are a main component of the enterocyte glycocalyx, whereas MUC2 is the most important gel-forming mucin that is secreted by goblet cells (Pelaseyed et al., 2014). MUC3 can be considered as a neutral mucin, while MUC2 can be considered as an acidic cysteine-rich mucin, due to their different aminoacid composition in proline, threonine and serine, (Kim, Gum Jr., Byrd, & Toribara, 1991). Thus, MUC3 stains positive for PAS, and MUC2 stains positive for AB. For the experiments, HT29-19A and HT29-16E cells were cultured without passaging for a tleast 12 days and then seeded into wells of 24-well plates at 2×105 cells/mL. After 2 weeks, the digested breads, diluted at 5% in DMEM (Maccaferri et al., 2010, 2012), were added to the cells and incubated for different times (2h, 4h and 24h). PAS staining system (Sigma, Inc) was used to evaluate neutral mucin (Bouhet & Oswald, 2005; Steedman, 1950). Briefly, cell monolayers were immersed in PAS solution for 5 min at room temperature followed by several rinses in dH2O. Cells were immersed subsequently in Schiff's reagent for 15min at room temperature, and washed in tap water. Cells were then counterstained in Gill's haematoxylin solution no. 3 (Sigma, UK) for 90s and again washed in tap water. Cell monolayers were allowed to dry and PAS staining assessed under the microscope. For the Alcian blue staining (Sigma, UK), the HT29-19A and HT29-16E cells were first fixed with chilled 95% ethanol / 5% glacial acetic acid for 10min, and then incubated with 1% Alcian blue / 3% acetic acid for 5 min at room temperature. The cells were subsequently washed three times with phosphate buffer (Sigma, UK) to remove any residual stain. Mucin production, assessed by the level of blue staining, was examined by light microscopy using an OLYMPUS BX43F microscope (Olympus Life Science, Tokyo, Japan). Images were acquired using Infinity Capture software. In addition, the pictures were also analysed by means of Photoshop and Image Tool software, which convert the images to binary mode: the dark blue stained cells turn black, and the light blue unstained cells become white. The percentage of black and white pixels is then calculated in order to compare the effect of the different treatments. Mucin production by Alcian blue staining was expressed as a percentage (%) of black pixels obtained from Image Tool software.

2.5. Measurement of Trans-Epithelial Electrical Resistance (TEER) in cell monolayers

In order to evaluate the effect of GFB and CB on the cell monolayers, specifically its integrity and permeability, TEER measurements were taken using a Millicell-ERS meter (Millipore, Billerica, MA, USA) connected to a pair of electrodes, following the manufacturer's standard procedure. HT29-16E cells were seeded on microporous Transwell® 6 well plates (Sigma,UK) at 2×105 cells/mL for 3 weeks at 37° C and 5% CO_2. HT29-19A non-mucus-secreting cells were used as a control. Cells were grown into polarized monolayers and TEER was measured at regular intervals (7, 10, 14, 18, 21days). Monolayers with a TEER > 250 Ωcm^2 were used for exposure to digested bread products. HT29-16E cell monolayers were exposed to digested breads (5%) for 24h at 37° C and 5% CO_2. The 24-hour time frame was established as the optimal time measurement to evaluate *in vitro* the effects of substances on cell barrier integrity of the small intestine mucosa (Barnett, Roy, McNabb, & Cookson, 2016; Damiano et al., 2018). The integrity of the polarized cell monolayers was determined before and after treatments by measuring the TEER. TEER values were standardized and reported as percent changes relative to the TEER at the beginning of the experiment. After the last TEER measurement, all supernatants and cell lysates were collected and processed for further analysis of MUC2 and MUC3 levels.

2*.6. Human MUC2 and MUC3 quantification by ELISA assays*

Human MUC2 (Abbexa, UK, cat abx055282) and MUC3 ELISA (Abbexa, UK, cat abx152398) kits were used to determine the level of these two types of mucins in the supernatants and cell lysates. Concentrations of MUC2 in cell culture supernatants and MUC3 in cell lysates were analysed by Human Mucin enzyme linked immunosorbent assays (ELISA) Kits (Abbexa Ltd, Cambridge, UK). Briefly, HT-29 16E cell supernatants were taken; cells were washed with PBS in order to take all non-adherent mucins and they were finally centrifuged at 6000 rpm for 20 min to remove the precipitant. Non-adherent MUC2 production was measured in this fraction. Regarding the MUC3 quantification, HT-29 16E cell monolayers free ofnon-adherent mucins were detached with trypsin (1.5mL) and collected by centrifugation at 4000 rpm for 10min to remove the supernatant. Then the cells were washed three times in ice-cold PBS, lysed by ultra-sonication and centrifuged to remove cellular debris. MUC3 sandwich ELISA was carried out on this fraction. Control analyses were also carried out.

2.7. Statistical analysis

For the MTT, Mucin2, Mucin3 and TEER assays, statistical analysis was performed using one-way ANOVA with a significance level set at 0.05, followed by Tukey multiple comparison test, in order to assess the effect of the different treatments. For the Alcian blue staining at different time-points, the statistical analysis was performed using two-way ANOVA, comparing the simple effects of treatment within each timepoint. All analyses were performed using GraphPad Prism Software (version 7.0, Inc., San Diego, CA, USA).

3. Results

3.1. Cytotoxic activity

Cytotoxicity of CB and GFB was evaluated by MTT assay in order to establish the optimal concentration to be used in the following experiments. Fig. 1 shows the percentage of viability of HT29-16E cells from healthy human models, after 24h incubation in the presence of the two different types of digested bread (1%, 5% and 10% v/v) compared to the control. The cytotoxicity activity data were expressed as a percentage of viable cells, in relation to the control wells, which represent 100% viability. It was observed that the treatments were not cytotoxic for the cells at any of the concentrations tested.

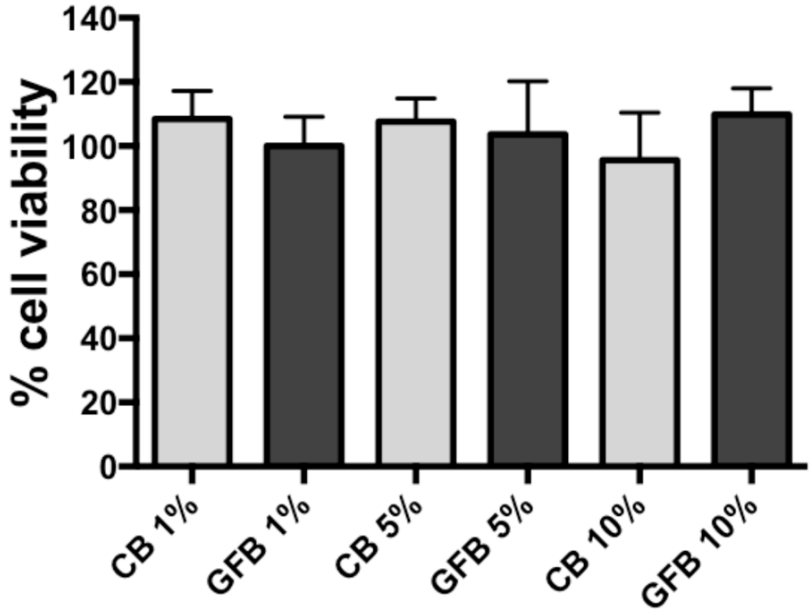

Fig. 1. Cell viability measured by MTT assay in HT29-16E cellsafter 24h of incubation with different concentrations of control bread (CB) and Gluten Friendly™ bread (GFB). Data are means of 3 separate experiments, each performed in 4 replicates (n = 12), and presented as mean ± SD. Statistical analysis was conducted by One-way ANOVA with Tukey post-hoc test ($P > 0.05$ vs. control).

3.2. *Assessment of mucin production by Periodic acid Schiff and Alcianblue staining*

Fig. 2 shows representative images of Alcian blue (Fig. 2A) and PAS (Fig. 2B) stainings of the HT29-16E cells (mucus-secreting) and of the HT29-19A cells (non-mucus-secreting) after 4h of incubation with the digested breads. The stainings depict common morphological patterns of mucus production by these cells. The HT29-19A non-mucus-secreting cell line did not present relevant levels of PAS and AB staining as expected. With regards to the HT29-16E cells (mucus-secreting) we can observe that in the GFB group, the levels of staining with AB (Fig. 2A) are higher than in both the CB group and the control. With regards to PAS staining (Fig. 2B) of HT29-16E cells (mucus-secreting), control cells (exposed to control solution resulting from in vitro digestion) showed levels of staining similar to the levels in CB treated cells. CB treated cells and control cells showed lower levels of PAS staining than GFB treated cells. Additionally, Fig. 2C provides a semi-quantitative analysis using Photoshop and Image Tool software, ofthe AB staining at 3 time-points (2h, 4h, and 24h), showing that the cells treated with CB and GFB had significantly higher levels of staining, expressed as percentage of pixels, in comparison with the control, after 4h and 24h treatments (see Fig. 2C).

3.3. Assessment of cells mucin production by ELISA

Human MUC2 (Abbexa, UK, cat abx055282) and MUC3 ELISA (Abbexa, UK, cat abx152398) kits were used to determine the level of these two types of mucins in the supernatants and cell lysates, respectively (Fig. 3). The cells treated with GFB had a significantly higher level of MUC2 (Fig. 3A) than the ones incubated with the Control ($P < 0.001$) or CB ($P < 0.01$). No significant differences were observed in MUC3 levels (Fig. 3B) among all different treatments.

3.4. Assessment of cells monolayer integrity by TEER measurement

The integrity of the cells monolayer before and after treatment with the digested breads was evaluated by the TEER. Fig. 4 reports the TEER values, expressed as % of the initial TEER. Both CB and GFB treated cells had significantly higher TEER values compared to control cells ($P < 0.01$ and $P < 0.001$, respectively). In addition, the cells treated with GFB had significantly higher TEER values than CB treated cells.

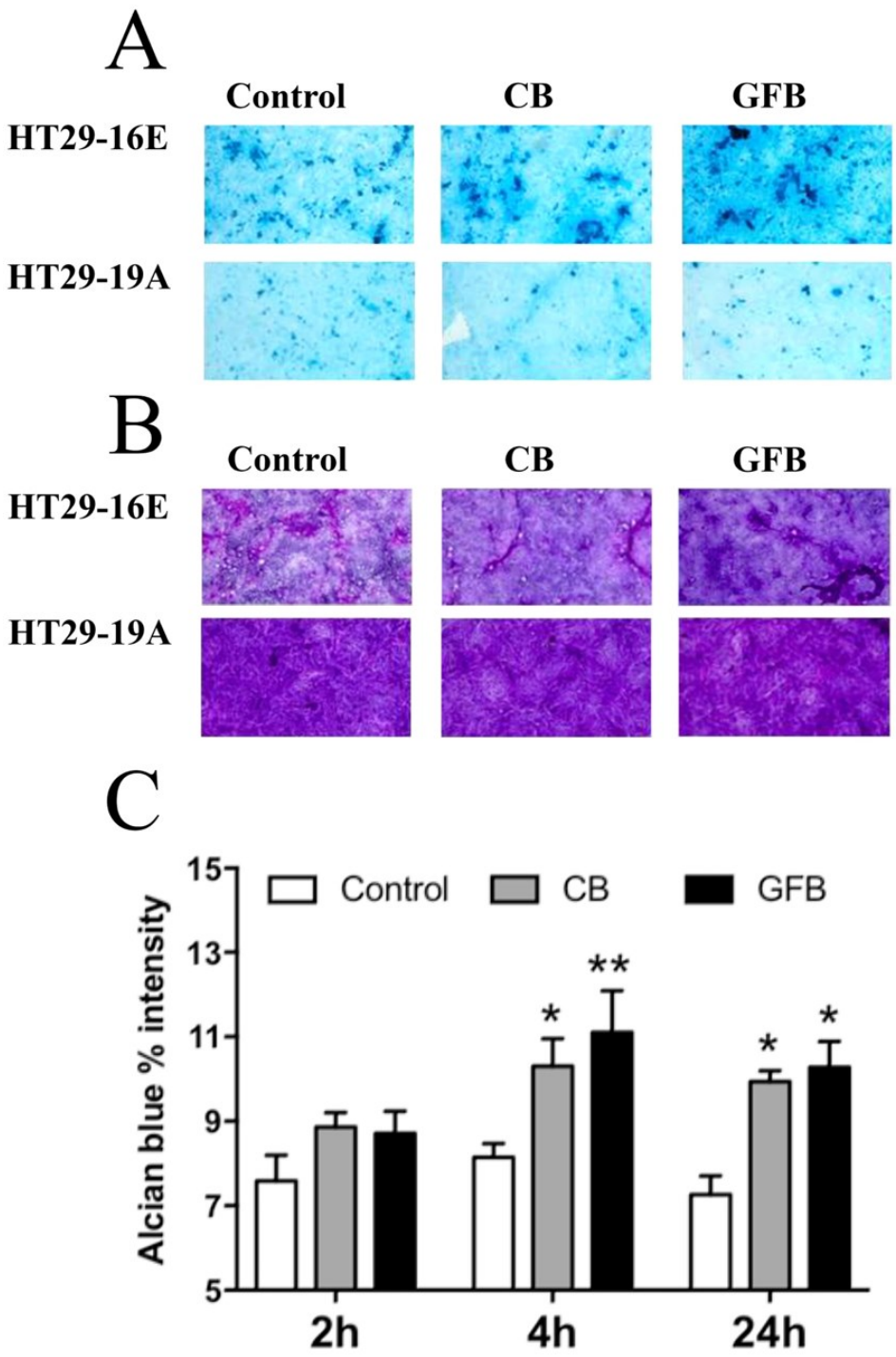

Fig. 2. Representative images of mucin staining in HT29-16E (mucus-secreting) and HT29-19A (non-mucus-secreting) cell lines after 4h of incubation with the digested breads: control bread (CB) and gluten-friendly bread (GFB) compared to a control cell monolayer. Acid mucins were visualized by Alcian Blue (AB) staining (x10 objective) (A) and neutral mucins were visualized by Periodic acid-Schiff (PAS) staining (×10 objective) (B). 3 separate experiments were performed, with 5 replicates for each experimental group (n = 15). Acid mucin production semi-quantitative analysis after 2h, 4h and 24h incubation of HT29-16E cells with control bread (CB) and gluten-friendly bread (GFB), compared to a control cell monolayer (C). The staining was performed with Alcian Blue and images were analysed using Photoshop and Image Tool. Data are means of 3 separate experiments, each performed in 5 replicates (n = 15), and presented as mean ± SD. Statistical analysis was conducted by One-way ANOVA with Tukey post-hoc test (* = $P < 0.05$ vs. control; ** = $P < 0.01$ vs. control).

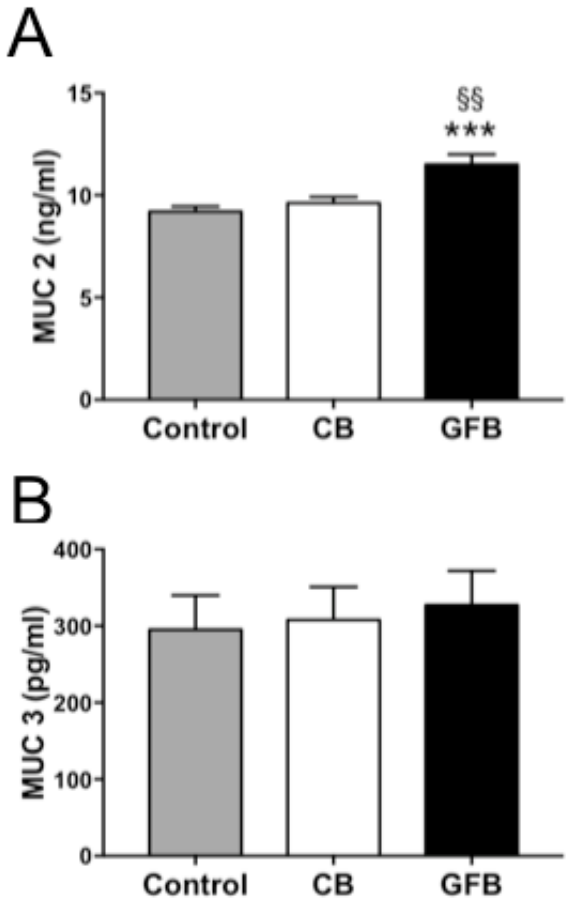

Fig.3. Quantification of specific mucin production by HT29-16E cells after 24h incubation with control bread (CB) and gluten-friendly bread (GFB), compared to a control cell monolayer. MUC2 levels (A) and MUC3 levels (B) were determined by ELISA. Data are means of 3 separate experiments, each performed in 4 replicates (n = 12), and presented as mean ± SD. Statistical analysis was conducted by One-way ANOVA with Tukey post-hoc test (*** = $P < 0.001$ vs. control; §§ = $P < 0.01$ vs. CB).

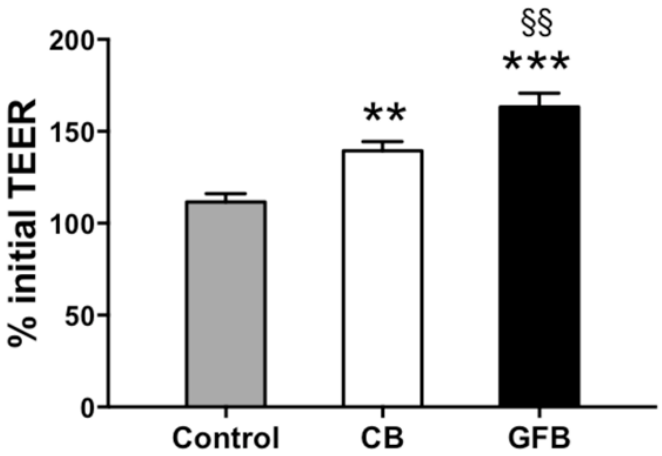

Fig. 4. Epithelial barrier function measured as Trans Epithelial Electrical Resistance (TEER). Data are shown as the % of initial TEER, in HT29-16E cells after 24h incubation with control bread (CB) and gluten-friendly bread (GFB), compared to a control cell monolayer. Data are means of 3 separate experiments, each performed in 3 replicates, 2 measurement for each replicate (n=18), and presented as mean ± SD. Statistical analysis was conducted by One-way ANOVA with Tukey post-hoc test (** = $P < 0.01$ vs. control; *** = $P < 0.001$ vs. control; §§ = $P < 0.01$ vs. CB).

4. Discussion

It is well-known that the intestine is the first organ exposed and affected by nutrients or antigens, following their ingestion (Maresca & Fantini, 2010; Scaldaferri, Pizzoferrato, Gerardi, Lopetuso, & Gasbarrini, 2012). Whereas specific membrane transporters allow absorption of beneficial compounds, the tight junctions and the mucus layer are responsible for the barrier function of the gut (Brandtzaeg, 2013; Pinton et al., 2015). Mucus plays major roles in the intestinal barrier function, and in the symbiosis with the microbiota. Perturbations of the composition and/or secretion of mucus are associated with diseases in animals and humans (Johansson, Sjövall & Hansson, 2013; Kim & Deng, 2008). Many studies have reported quantitative and qualitative abnormalities, of mucin gene expression in gastrointestinal diseases (Hafez, 2012). Previous studies have shown that GFB, produced from flours treated with the novel, patented temperature-based process (Lamacchia et al., 2016) is able to modulate *in vitro*, the gut microbiota function in both healthy and coeliac donors (Bevilacqua et al., 2016; Costabile et al., 2017). The main hypothesis of this study was to determine whether GFB could have beneficial effects on mucus production and intestinal epithelial barrier *in vitro*. To study the effect of GFB on intestinal mucus, we used the HT29-16E mucus-secreting cells that, like normal goblet cells, produce mucins (Augeron & Laboisse, 1984; Maoret et al., 1989). Confluent HT29-16E monolayers secrete a dense mucus gel and represent an established healthy human model to study goblet cell mucus secretion *in vitro*. Importantly, ex *vivo* experiments performed on intestinal explants from pigs confirmed *in vitro* data, demonstrating the high predictive value of the HT29-16E cells as a health model of intestinal goblet cells (Pinton et al., 2015). It has been previously reported that granules positive for Alcian blue contain (Behrens, Stenberg, Artursson & Kissel, 2001) non-sulfated acidic mucins or sialomucin (pH 2.5), as well as sulphated mucins or sulphomucins (pH 1.0), and also that PAS-positive goblet cells secrete neutral mucins (Trevizan et al., 2016). Studies about mucin secretion have proven challenging in the past, as mucins are difficult to purify. At the same time, both chemical or radioactive labelling have limitations because labelling is restricted to a single attribute, such as a monosaccharide or amino acid, which is not exclusive to mucins, or necessarily evenly distributed among them (Forstner, 1995). The data presented here demonstrated that GFB does not affect cell viability, as indicated by the MTT assay, but does increase the secretion of mucins as observed after PAS and Alcian blue staining, in comparison with the control cells.

Whereas a rise in mucus production is indicative of a healthy response of the intestinal epithelia (Forstner, 1995), reduced mucus production is consistently observed in Inflammatory Bowel Diseases, such as ulcerative colitis and Crohn disease (Lammers et al., 1994; Makkink et al., 2002). Alteration of mucin production has also been reported in CD patients (Bischoff et al., 2014; Einerhand

et al., 2002; Strugala, Dettmar & Pearson, 2008) and has been linked to the onset of CD (Boltin, Perets, Vilkin & Niv, 2013).

To better investigate the effect of GFB on mucin production, we used two specific MUC2 and MUC3 Human enzyme linked immunosorbent assays (ELISA) that allowed us to quantify the two mucins, and discriminate the inner layer attached firmly to the epithelial surface, and the outer layer non-adherent mucus (Augeron & Laboisse, 1984; Crabtree, Heatley & Losowsky, 1989). MUC3 is a membrane bound adherent mucin, while MUC2 is a gel-forming mucin secreted on the outer or non-adherent mucus layer (Augenlicht, Augeron, Yander & Laboisse, 1987).

Thus, MUC2 ELISAdata support the finding that GFB induces higher production of MUC2 than the control and CB. No significant difference was observed in MUC3 secretion throughout different experiment conditions. Arike and Hansson (2004) explain that MUC2 mucins have a protective role owing to their o-glycosylation density, forming a hydrophilic diffusion barrier with its bound water. Furthermore, MUC2 also provides food for commensal bacteria (Byrd & Bresalier, 2004) and prevents pathogens from reaching the glycocalyx beneath, which is devoid of microbes (Arike & Hansson, 2004; Johansson et al., 2008). It is important to highlight that MUC2 does not only constitute a nonspecific physical barrier, but also significantly affects the immunogenicity of gut antigens by delivering tolerogenic signals to dendritic cells, via the intestinal epithelial cells, and therefore enhancing gut homeostasis (Johansson et al., 2013; Shan et al., 2002). A reduced MUC2 production has indeed been associated to a range of gastrointestinal inflammatory states, including ulcerative cholitis (Makkink et al., 2002; Shan et al., 2013). The data presented here support the potential of GFB to maintain and/or enhance gut homeostasis and intestinal barrier function, by maintaining higher mucin levels than control and CB treated cells. Indeed, GFB triggers MUC2 production to a higher extent, potentially contributing to create a stronger intestinal barrier. It is also worth underlining that innate immunity has been considered as another possible key element in the development of CD (Van Klinken, Van der Wal, Einerhand, Büller, & Dekker, 1999), which is characterized by imbalances of the intestinal microbiota composition, thus suggesting a role of intestinal microbiota in this pathology. The mode by which GFB stimulates mucin production requires further investigation. Results collected so far suggest that exposure of hydrated wheat caryopses to the Gluten Friendly™ technology induces a different spatial conformation of the amino acid sequences and a rearrangement of the secondary and tertiary structure of gluten proteins (Lamacchia et al., 2016). Furthermore, it has been postulated that such rearrangement of the gluten protein structure exposes positive charges, namely cationic residues, and could explain the novel effects of GFB on bacteria and probiotics (Bevilacqua et al., 2016). Given that secretion is triggered by a wide array of bioactive factors, including cholinergic agonist, hormones (neuropeptides), microbes and

microbial products (peptides), inflammatory cytokines, and reactive oxygen and nitrogen species (Kim & Ho, 2010), the cationic peptides in GFB could also be acting as mucin segretagogues on HT29-16 E mucus-secreting cells.

TEER values for human small intestine vary from 50 to 100 Ω/cm^2. However, in this study, the integrity of the monolayer was confirmed by TEER > 200 Ω/cm^2 (Martínez-Maqueda et al., 2015, chap. 11). Variations in TEER measurements may arise due to differing culture parameters, such as the passage number of the cells; the age and the stage of differentiation of the cells; the type of culture medium used; the seeding density of the cells and the type of support the cells are cultured on (Sambuy,2005). Therefore, inspite of some limitations of the *in vitro* cell model (Martínez-Maqueda et al., 2015, chap. 11; Sambuy, 2005), this study indicated the potential for GFB to improve the intestinal cellular barrier integrity, as indicated by the significantly higher TEER increase, compared to both control and CB. Even though the exact interactions and contributions of the intestinal mucosa are not clearly defined, an increased TEER signal indicates increased intestinal barrier function. Mucins are a key extracellular component of the intestinal barrier (Rossi&Schwartz,2010). It could be assumed that the increased trans-epithelial electrical resistance is mainly due to the increase in the gel-forming MUC2 secretion. However, it should be noted that the glycocalyx and tight junction proteins are also known to be key contributors (Lee, 2015).

Abnormal bacterial adherence and internalization by epithelial cells have been reported in CD Patients (Drago et al., 2006; Rossi & Schwartz, 2010). *In vitro* studies using Caco-2 cells and IEC6 have shown that tight junction protein interactions are compromised by gluten (Rossi & Schwartz, 2010). This leads to the rearrangement of the cytoskeleton, and increases monolayer permeability (Drago et al., 2006). With recent knowledge that adherent mucosa-associated bacteria play a critical role in IBS (Irritable Bowel Syndrome) and colitis associated colorectal cancers, there is increased understanding that an abnormal interaction between epithelium and bacteria also exists. There is now scientific consensus regarding the importance of gut microbiome in health and disease (Byrd & Bresalier, 2004; Ulluwishewa et al., 2011) and there are many factors that influence its composition (Nicholson et al., 2012; Ulluwishewa et al., 2011).

The gut microbial dysbiosis seen in CD patients has been found to dramatically affect the host physiology (Yatsunenko et al., 2012). Recently, the effect of GFB on the gut microbiota of both healthy and CD patients has been investigated (Bevilacqua et al., 2016; Costabile et al., 2017) and it has been shown that GFB positively modulated the complex bacterial ecosystem with an increase in numbers of health-promoting beneficial bacteria (Costabile et al., 2017).

Additionally, GFB prolonged the survival of *Lactobacillus acidophilus* and had antibacterial effects towards *Staphylococcus aureus* and *Salmonella Typhimurium* (Bevilacqua et al., 2016). Other studies

have found that *L. acidophilus* counteracts inhibition of butyrate uptake in intestinal epithelial cells by enteropathogenic *E. coli* (Krishnan, Alden & Lee, 2015).

It has been reported that the intestinal microbiota may modulate goblet cell function and the intestinal mucus layer (Kumar, Rajendran, Kumar, Hamwieh, & Baum, 2015). Therefore, taken together with the outcome of the present study, intestinal epithelium mucus production may be increased not only by a direct effect of GFB itself, but also as a consequence of gut microbiota modulation triggered by GFB. Further research is, however, required to assess whether such functionality is also maintained *in vivo*, particularly in coeliac subjects. It could be concluded that GFB has the potential to induce mucin secretion by intestinal epithelial cells and to improve intestinal epithelial barrier function. Thus *in vivo* studies are recommended to confirm the *in vitro* outcome presented in this study. If confirmed, such observed potential may effectively contribute to consequent benefits, such as higher gut barrier defense, decreased susceptibility to infections and better absorption regulation, thus helping to redress such disturbances in chronic inflammatory intestinal diseases.

Author contributions

CL has conceptualized the study; CL, AC and GC have designed the study; CL, LL and ID applied the Gluten Friendly™ temperature treatment on wheat kernels for the production and characterization of GFB; TBM, DM and MEH have carried out the cell culture experiments; GC and AC carried out the statistical analysis; CL obtained funding; CL, AC and GC have supervised the study and have edited and validated the data analysis; CL has interpreted the data; AC and GC drafted the manuscript; CL has critically revised the manuscript for important intellectual content; CL, AC and GC have edited and approved the final manuscript.

Conflict of interests

Carmen Lamacchia declares to be the inventor of the following patents “Method for the detoxification of gluten proteins from grains of cereals. Patent Cooperation Treaty PCT/IB2013/000797” and “Methods forthe detoxification of gluten proteins from grains of cereals and related medical uses. Italian priority patent n° 102015000084813 filed on 17.12.15.”

The authors declare no conflict of interest. Casillo Group was a commercial source; however, it had no role in the design of the basic patent of this research (Gluten Friendly™ temperature-based process), and did not play any role in the design of this research.

Ethics statement

The cellular *in vitro* model used in this study was a commercially available model. In this study we did not used any kind of human samples to require approval of Ethics Committee.

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References

Alaedini, A., & Green, P. H. (2005). Narrative review: Celiac disease: Understanding a complex autoimmune disorder. Annals of Internal Medicine, 142, 289–298.

Arike, L., & Hansson, G. C. (2004). The densely O-glycosylated MUC2 mucin protects the intestine and provides food for the commensal bacteria. Journal of Molecular Biology, 428, 3221–3229.

Augenlicht, L. H., Augeron, C., Yander, G., & Laboisse, C. (1987). Overexpression of ras in mucus-secreting human colon carcinoma cells of low tumorigenicity. Cancer Research, 47, 3763–3765.

Augeron, C., & Laboisse, C. L. (1984). Emergence of permeability differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate. Cancer Research, 44, 3961–3969.

Barnett, A. M., Roy, N. C., McNabb, W. C., & Cookson, A. L. (2016). Effect of a semipurified oligosaccharide-enriched fraction from caprine milk on barrier integrity and mucin production of co-culture models of the small and large intestinal epithelium. Nutrients, 8, 267.

Bascunan, K. A., Vespa, M. C., & Araya, M. (2017). Celiac disease: Understanding the gluten- free diet. European Journal of Nutrition, 56, 449–459.

Behrens, I., Stenberg, P., Artursson, P., & Kissel, T. (2001). Transport of lipophilic drug molecules in a new mucus-secreting cell culture model based on HT29-MTX cells. Pharmacology Research, 18, 1138–1145.

Bevilacqua, A., Costabile, A., Bergillos-Meca, T., Gonzalez, I., Landriscina, L., Ciuffreda, E., ... Lamacchia, C. (2016). Impact of gluten-friendly bread on the metabolism and function of in vitro gut microbiota in healthy human and coeliac subjects. PloS One, 11, e0162770.

Bischoff, S. C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J. D., Serino, M., ... Wells, J. M. (2014). Intestinal permeability – A new target for disease prevention and therapy. BMC Gastroenterology, 14, 189.

Boltin, D., Perets, T. T., Vilkin, A., & Niv, Y. (2013). Mucin function in inflammatory bowel disease: An update. Journal of Clinical Gastroenterology, 47, 106–111.

Bouhet, S., & Oswald, I. P. (2005). The effects of mycotoxins, fungal food contaminants, on the intestinal epithelial cell-derived innate immune response. Veterinary Immunology and Immunopathology, 108, 199–209.

Brandtzaeg, P. (2013). Gate-keeper function of the intestinal epithelium. Beneficial Microbes, 4, 67 –82.

Byrd, J. C., & Bresalier, R. S. (2004). Mucins and mucin binding proteins in colorectal cancer. Cancer Metastasis Review, 23, 77 –99.

Costabile, A., Bergillos-Meca, T., Landriscina, L., Bevilacqua, A., Gonzalez-Salvador, I., Corbo, M.R., Lamacchia, C. (2017). An *in vitro* fermentati on study on the effects of Gluten Friendly™ bread on microbiota and short chain fatty acids of fecal samples from healthy and celiac subjects. Frontiers in Microbioliogy, 8, 1722.

Crabtree, J. E., Heatley, R. V., & Losowsky, M. S. (1989). Glycoprotein synthesis and secretion by cultured small intestinal mucosa in coeliac disease. Gut, 30, 1339–1343.

Damiano, S., Sasso, A., De Felice, B., Di Gregorio, I., La Rosa, G., Lupoli, G. A., ... Santillo, M. (2018). Quercetin increases MUC2 and MUC5AC gene expression and secretion in intestinal goblet cell-like LS174T via PLC/PKCα/ERK1-2 pathway. Frontiers in Physiology, 9, 357.
Davis, J. M. (2002). Basic cell culture: A practical approach (2nd ed.). Oxford, UK: University Press 381.
Diebel, L. N., Liberati, D. M., & Hall-Zimmerman, L. (2011). H2 blockers decrease gut mucus production and lead to barrier dysfunction in vitro. Surgery, 150, 736–743.
Drago, S., El Asmar, R., Di Pierro, M., Grazia Clemente, M., Sapone, A. T. A., Thakar, M., ...Fasano, A.(2006). Gliadin, zonulin and gut permeability: Effects on celiac and non celiac intestinal mucosa and intestinal cell lines. Scandinavian Journal of Gastroenterology, 41, 408–419.
Einerhand, A. W., Renes, I. B., Makkink, M. K., van der Sluis, M., Buller, H. A., & Dekker, J. (2002). Role of mucins in inflammatory bowel disease: Important lessons from experimental models. European Journal of Gastroenterology & Hepatology, 14, 757–765.
Forstner, G. (1995). Signal transduction, packaging and secretion of mucins. Annual Review of Physiology, 57, 585–605.
Fromm, M., Krug, S. M., Zeissig, S., Richter, J. F., Rosenthal, R., Schulzke, J. D., & Günzel, D. (2009). High-resolution analysis of barrier function. Annals of the New York Academy of Sciences, 1165, 74 –81.
Hafez, M. M. (2012). Upregulation of intestinal mucin expression by the probiotic bacterium E. coli Nissle 1917. Probiotics and Antimicrobial Proteins, 4, 67 –77.
Hall, N. J., Rubin, G. P., & Charnock, A. (2013). Intentional and inadvertent non-adherence in adult coeliac disease. A cross-sectional survey. Appetite, 68, 56 –62.
Hallert, C., Grant, C., Grehn, S., Granno, C., Hulten, S., Midhagen, G., ... Valdimarsson, T. (2002). Evidence of poor vitamin status in coeliac patients on a gluten-free diet for10 years. Alimentary Pharmacology & Therapeutics, 16, 1333–1339.
Jarry, A., Merlin, D., Hopfer, U., & Laboisse, C. L. (1994). Cyclic AMP-induced mucin exocytosis is independent of Cl- movements in human colonic epithelial cells (HT29Cl.16E). Journal of Biochemistry, 304, 675–678.
Johansson, M. E. V., Gustafsson, J. K., Holmén-Larsson, J., Jabbar, K. S., Xia, L., Xu, H., Hansson, G. C., et al. (2013). Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. Gut, 63, 281–291.
Johansson, M. E., Phillipson, M., Petersson, J., Velcich, A., Holm, L., & Hansson, G. C. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proceedings of the National Academy of Sciences of the United States of America, 105, 15064–15069.
Johansson, M. E., Sjövall, H., & Hansson, G. C. (2013). The gastrointestinal mucus system in health and disease. Home: Nature Reviews Gastroenterology & Hepatology, 10, 352–361.
Kim, Y. S., & Deng, G. (2008). Aberrant expression of carbohydrate antigens in cancer: The role of genetic and epigenetic regulation. Gastroenterology, 135, 305–309.
Kim, Y. S., Gum, J. R., Jr., Byrd, J. C., & Toribara, N. W. (1991). The structure of human intestinal apomucins. The American Review of Respiratory Disease, 144, S10–S14.
Kim, Y. S., & Ho, S. B. (2010). Intestinal goblet cells and mucins in health and disease: Recent insights and progress. Current Gastroenterology Reports, 12, 319–330.
Krishnan, S., Alden, N., & Lee, K. (2015). Pathways and functions of gut microbiota metabolism impacting host physiology. Current Opinion in Biotechnology, 36, 137–145.
Kumar, S., Rajendran, K., Kumar, J., Hamwieh, A., & Baum, M. (2015). Current knowledge in lentil genomics and its application for crop improvement. Frontiers in Plant Science, 6, 78 .
Lamacchia, C., Landriscina, L., & D'Agnello, P. (2016). Changes in wheat kernel proteins induced by microwave treatment. Food Chemistry, 197, 634–640.
Lammers, K. M., Jansen, J., Bijlsma, P. B., Ceska, M., Tytgat, G. N., Laboisse, C. L., & van Deventer, S. J. (1994). Polarised interleukin 8 secretion by HT 29/19A cells. Gut, 35, 338–342.
Landriscina, L., D'Agnello, P., Bevilacqua, A., Corbo, M. R., Sinigaglia, M., & Lamacchia, C. (2017). Impact of gluten-friendly™ technology on wheat kernel endosperm and gluten protein structure in seeds by light and electron microscopy. Food Chemistry, 221, 1258–1268.
Lee, S. H. (2015). Intestinal permeability regulation by tight junction: Implication on inflammatory bowel diseases. Intestinal Research, 13, 11 –18.
Lundin, K. E., & Sollid, L. M. (2014). Advances in coeliac disease. Current Opinion in Gastroenterology, 30, 154–162.

Maccaferri, S., Klinder, A., Cacciatore, S., Chitarrari, R., Honda, H., Luchinat, C., ... Costabile, A. (2012). In vitro fermentation of potential prebiotic flours from natural sources: Impact on the human colonic microbiota and metabolome. Molecular Nutrition and Food Research, 56, 1342–1352.
Maccaferri, S., Vitali, B., Klinder, A., Kolida, S., Ndagijimana, M., Laghi, L., ... Costabile, A. (2010). Rifaximin modulates the colonic microbiota of patients with Crohn's disease: An in vitro approach using a continuous culture colonic model system. Journal of Antimicrobial Chemotherapy, 65, 2556–2565.
Makkink, M. K., Schwerbrock, N. M., Mähler, M., Boshuizen, J. A., Renes, I. B., Cornberg, M., ... Dekker, J. (2002). Fate of goblet cells in experimental colitis. Digestive Diseases and Sciences, 47, 2286–2297.
Maoret, J. J., Font, J., Augeron, C., Codogno, P., Bauvy, C., Aubery, M., & Laboisse, C. L. (1989). A mucus-secreting human colonic cell line. Purification and partial characterization of the secreted mucins. Journal of Biochemestry, 258, 793–799.
Maresca, M., & Fantini, J. (2010). Some food-associated mycotoxins as potential risk factors in humans predisposed to chronic intestinal inflammatory diseases. Toxicon, 56, 282–294.
Martínez-Maqueda, D., Miralles, B. & Recio, I. (2015). HT29 cell line. In K. Verhoeckx et al. (Eds.), The impact of food bio-actives on gut health (pp. 117–118).
Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., & Pettersson, S. (2012). Host-gut microbiota metabolic interactions. Science, 336, 1262–1267.
Olivares, M., Neef, A., Castillejo, G., Palma, G. D., Varea, V., Capilla, A., ... Sanz, Y. (2015). The HLA-DQ2 genotype selects for early intestinal microbiota composition in infants at high risk of developing coeliac disease. Gut, 64, 406–417.
Pelaseyed, T., Bergstrom, J. H., Gustafsson, J. K., Ermund, A., Birchenough, G. M. H., Schatte, A., Hansson, G. C. (2014). The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. Immunological Reviews, 260,8 –20.
Peterson, L. W., & Artis, D. (2014). Intestinal epithelial cells: Regulators of barrier function and immune homeostasis. Nature Review and Immunology, 14, 141–153.
Pinton, P., Graziani, F., Pujol, A., Nicoletti, C., Paris, O., Ernouf,P., Maresca, M. (2015). Deoxynivalenol inhibits the expression by goblet cells of intestinal mucins through a PKR and MAP kinase dependent repression of the resistin-like molecule. Molecular Nutrition and Food Research, 59, 1076–1087.
Rossi, M., & Schwartz, K. B. (2010). Editorial: Celiac disease and intestinal bacteria: Not only gluten? Journal of Leukocyte Biology, 87, 749–751.
Sambuy, Y. (2005). The Caco-2 cell line as a model of the intestinal barrier: Influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biology and Toxicology, 21,1 –26.
Scaldaferri, F., Pizzoferrato, M., Gerardi, V., Lopetuso, L., & Gasbarrini, A. (2012). The gut barrier: New acquisitions and therapeutic approaches. Journal of Clinical Gastroenterology, 46, S12–S17.
Schiller, C. J., Klainz, A., Mynett, K., & Gescher, A. (1992). Assessment of viability of hepatocytes in suspension using the MTT assay. Toxicolology in Vitro, 6, 575–578.
Schulzke, J. D., Bentzel, C. J., Schulzke, I., Riecken, E. O., & Fromm, M. (1998). Epithelial tight junction structure in the jejunum of children with a cute and treated celiacs prue. Pediatric Research, 43, 435–441.
Schumacher, U., Duku, M., Katoh, M., Jörns, J., & Krause, W. J. (2004). Histochemical similarities of mucins produced by Brunner's glands and pyloric glands: A comparative study. The Anatomical Record Part A, Discoveries in Molecular, Cellular, and Evolutionary Biology, 278, 540–550.
Schumann, M., Günzel, D., Buergel, N., Richter, J. F., Troeger, H., May, C., Schulzke, J. D. (2012). Cell polarity-determining proteins Par-3 and PP-1 are involved in epithelial tight junction defects in coeliac disease. Gut, 61, 220–228.
Schumann, M., Siegmund, B., Schulzke, J., & Fromm, M. (2016). Celiac disease: Role of the epithelial barrier. Cellular and Molecular Gastroenterology and Hepatology, 3, 150–162.
Shan, M., Gentile, M., Yeiser, J.R., Walland, A.C., Bornstein, V.U., Chen, K., Cerutti, A. (2013). Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. Science, 342, 447–453.
Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G. M., Khosla, C. (2002). Structural basis for gluten intolerance in celiac sprue. Science, 297, 2275–2279.
Shannahan, S., & Leffler, D. A. (2017). Diagnosis and updates in celiac disease. Gastrointestinal Endoscopy Clinics of North America, 27, 79 –92.
Shepherd, S. J., & Gibson, P. R. (2013). Nutritional inadequacies of the gluten-free diet in both recently-diagnosed and long-term patients with coeliac disease. Journal of Human Nutrition and Dietetics: The Official Journal of the British Dietetic Association, 26, 349–358.

Smecuol, E., Bai, J. C., Vazquez, H., Kogan, Z., Cabanne, A., Niveloni, S., Meddings, J. B. (1997). Gastrointestinal permeability in celiac disease. Gastroenterology, 112, 1129–1136.
Steedman, H. F. (1950). Alcian blue 8GS: A new stain for mucin. The Quarterly Journal of Microscopical Science, 91, 477–479.
Strugala, V., Dettmar, P. W., & Pearson, J. P. (2008). Thickness and continuity of the adherent colonic mucus barrier in active and quiescent ulcerative colitis and Crohn's disease. International Journal of Clinical Practice, 62, 762–769.
Thompson, T., & Simpson, S. (2015). A comparison of gluten levels in labeled gluten-free and certified gluten-free foods sold in the United States. European Journal of Clinical Nutrition, 69, 143–146.
Tootian, Z., Sadeghinezhad, J., Sheibani, M.T., Fazelipour, S., DeSordi, N., & Chiocchetti, R. (2013). Histological and mucin histochemical study of the small intestine of the Persian squirrel (Sciurus anomalus). Anatomical Science International, 88, 38 –45.
Trevizan, A. R., Vicentino-Vieira, S. L., da Silva Watanabe, P., Góis, M. B., de Melo Gde, A., Garcia, J. L., Sant'Ana Dde, M. (2016). Kinetics of acute infection with Toxoplasma gondii and hispopathological changes in the duodenum of rats. Experimental Parasitology, 165, 22 –29.
Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. Nature Review and Immunology, 9, 799–809.
Ulluwishewa, D., Anderson, R. C., McNabb, W. C., Moughan, P. J., Wells, J. M., & Roy, N. C. (2011). Regulation of tight junction permeability by intestinal bacteria and dietary components. The Journal of Nutrition, 141, 769–776.
Van Klinken, B. J., Van der Wal, J. W., Einerhand, A. W., Büller, H. A., & Dekker, J. (1999). Sulphation and secretion of the predominant secretory human colonic mucin MUC2 in ulcerative colitis. Gut, 44, 387–393.
Wells, J. M., Brummer, R. J., Derrien, M., MacDonald, T. T., Troost, F., Cani, P. D., Garcia-Rodenas, C. (2016). Homeostasis of the gut barrier and potential biomarkers. American Journal of Physiology-Gastrointestinal and Liver Physiology, 312, G171–G193.
Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Gordon, J.I. (2012). Human gut microbiome viewed across age and geography. Nature, 486, 222–227.

Chapter 3

The Impact of Gluten Friendly Flour on the Functionality of an Active Drink: Viability of Lactobacillus acidophilus in a Fermented Milk

The Impact of Gluten Friendly Flour on the Functionality of an Active Drink: Viability of Lactobacillus acidophilus in a Fermented Milk

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Barbara Speranza, Antonio Bevilacqua, Daniela Campaniello, Milena Sinigaglia, Daniela Musaico, Maria R. Corbo* and Carmela Lamacchia

Department of the Science of Agriculture, Food and Environment, University of Foggia, Foggia, Italy

*** Correspondence**:
Maria R. Corbo
mariarosaria.corbo@unifg.it

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Abstract

The Gluten Friendly™ Technology is an innovative method that induces structural changes in gluten proteins. In this paper a synbiotic fermented milk, containing LactobacillusacidophilusLa-5andGlutenFriendlyFlour(GFF), was proposed. A mixture design was used to combine flour, temperature and probiotic to study the effects of these variables on the acidification. The experiments were done on both GFF and control flour (CF). Thus, the following conditions were chosen to produce the fermented milk: L. acidophilus at 6.5 log cfu/ml; flour at 2.5 g/l; temperature at 37°C. Then, the fermented milk was produced and stored at 4°C for 90 days. The most important result was the positive effect of GFF on the viability of the probiotic, with a prolongation of the shoulder length to 20 days (12–13 days in the control). Moreover, GFF did not act on the sensory scores and on the physico-chemical parameters.

Keywords: synbiotic, Gluten Friendly, acidification, shoulder length, desirability

INTRODUCTION

Today the demand for healthy products is continuously increasing and the food industry has been showing interest and marketing functional foods, i.e., foods able to provide health benefits as they include basic nutrients and compounds reducing the risk of several diseases (González Fabre, 2008). Synbiotic foods are generally considered functional foods because they beneficially affect the host by improving the survival and implantation of probiotic microorganisms in the gastrointestinal tract by selectively stimulating their growth and/or the metabolism (Cencic and Chingwaru, 2010). Even if there is not a general consensus toward the definition of synbiotic foods, they could be defined as products containing a combination of probiotics and prebiotics that can act synergistically to modulate the intestinal microbiota and positively impact on people's health (Gotteland, 2010). So that probiotics exert health effects, the recommended minimum level of viable cells has been suggested to be between $10^6 – 10^7$ cfu/ml at the moment of consumption (Silva et al., 2015) and prebiotics have shown good results in helping probiotics to maintain their viability and functionality throughout all food processing steps (Akinetal., 2007; Cruzetal., 2009).

FAO/WHO (2006) defines "*prebiotics as a non-viable food component that confer health benefit(s) on the host associated with modulation of the microbiota*"; in general, prebiotics are carbohydrate ingredients of different origin: breast milk, soybeans, inulin sources (like Jerusalem artichoke, chicory roots etc.), raw oats, unrefined wheat, unrefined barley, yacon, and non-digestible oligosaccharides [oligofructose, and (trans) galacto-oligosaccharides (GOS) (Pokusaeva et al., 2011)]. Recently other sources (ß-glucans, ulvan, etc.) have been explored for their potential benefits as prebiotics (Saulnier et al., 2009) and this remains an active area of research. From a technological point of view, the addition of prebiotic to foods has been demonstrated to improve sensory characteristics such as taste and texture (Al-Sheraji et al., 2013); some evidences of enhancements of the stability of foams, emulsions, and mouth feel in a vast range of food applications like dairy and baking products have been also reported (Al-Sheraji et al., 2013).

In 2013 a new and innovative method (Gluten Friendly^{TM}) has been developed (PCT/IB2013/000797) (Lamacchia et al., 2013, 2015); it consists into the application of microwave energy for few seconds to hydrated wheat kernels. Due to some structural modifications to endosperm components, the immunogenicity of the most common epitopes involved in celiac disease was reduced *in vitro* (Lamacchia et al., 2016), but the nutritional and technological properties necessary to process flour into bread, pasta, and other baked goods remain unchanged. Additional researches (Bevilacqua et al., 2016b) found that bread produced with Gluten Friendly Flour (GFF) was able to modify the qualitative-quantitative composition of gut microbiota (bifidogenic effect and on the growth of lactobacilli in the gut microbiota in a complex system).

The probiotic potential of *L. acidophilus* La-5 was investigated and assessed in the past. Brasili et al. (2013) found that the supplementation of this probiotic induced a positive modulation of urinary and fecal metabolic profiling thus suggesting a possible effect on the prevention and/or the reduction of age-related metabolic dysfunction, while Zarrati et al. (2013) reported a significant modulation on T-cell subset specific gene expression in peripheral blood mononuclear cells among overweight and obese individuals. Other probiotic effects include the inhibition of *Escherichia coli* O157:H7 (Medellin-Peña and Griffiths, 2009), and the reduction of *Streptococcus mutans* in saliva (Bafna et al., 2018).

The strain can be successfully inoculated and supplemented with different foods, among others fruit-based ice-cream (Senanayake et al., 2013), blue cheese (Zadernowska et al., 2015), or loaded in microcapsules (Gebara et al., 2013; Ranadheera et al., 2015).

A preliminary research (Bevilacqua et al., 2016b) showed the potential of the combination of GF products with *L. acidophilus*, as the survival time of the probiotics was prolonged by 15–20h at 37°C and in a minimal medium.

Considering all these data, the aim of the present study was to design a synbiotic milk, fermented with *L. acidophilus* La5 and containing GFF as a beneficial component to improve the viability of the probiotic in real conditions and design a new functional food with an improved survival of *L. acidophilus* La-5. The research was divided into two different steps: (1) a product optimization to choose the optimal conditions to design the active drink, in terms of level of inoculum, temperature and amount of GFF; (2) validation at laboratory level with the production of a functional fermented milk and the evaluation of its microbiological, physico-chemical and sensorial quality during refrigerated storage for 90 days.

MATERIAL AND METHODS

Product Optimization

Microorganism, Flour and Milk

Lactobacillus acidophilus La-5 was purchased from Chr. Hansen (Hørsholm, Denmark). Prior each assay, the strain was grown in MRS broth (Oxoid, Basingstoke, United Kingdom) at 37°C for 24 h and then centrifuged at 4000g for 10 min; the supernatant was discarded, and the pellet was suspended in sterile distilled water.

The GFF was prepared as follow: namely, grain of wheat was treated according to the patented method PCT n. PCT/IB2013/000797, further improved (Italian priority patent n. 102015000084813. Method for the detoxification of gluten proteins from grains of cereals and related medical uses filed

on 17th December 2015. Inventor: Lamacchia C.). Specifically, 100 g of cleaned wheat grains was dampened until reaching 15– 18% humidity, which was measured by a halogen thermal balance (Mettler Toledo, HB43-S, Switzerland), and subjected to rapid heating via microwaves (De'Longhi, Italy; approximately 1min between 1000 and 750 watts), followed by slow evaporation of the water. The rapid heating and subsequent slow evaporation of the water was repeated until reaching a final temperature of 80–90°C, which was measured by a thermal camera (Fluke, i20 Model, Italy), and a moisture degree of 13–13.5% in the wheat grains.

After microwave treatment, the wheat kernels were cooled and dried at room temperature (24°C) for 12–24 h and then ground using an automatic laboratory mill MCKA (Bühler AG, Uzwil, Switzerland; diameter of grid 118–180 mm) (Bevilacqua et al., 2016b).

The flour produced by milling caryopses that had not been treated with microwaves was called control flour (CF). The particle size of the GFF and the CF used was in the range of 100 to 200 μm.

For all assays, fresh whole pasteurized homogenized cow's milk (3.35 g/l protein; 5.00 g/l carbohydrates; 3.75 g/l fats) was used. Before each experiment, the viable count of milk was assessed to check that lactobacilli were below the detection limit (standard plate count).

Experimental Design

The optimization of the production of the synbiotic fermented milk was performed through a mixture design, called simplex centroid design: this kind of design involves three different variables; each variable is usually set at three different levels, identified with the code 0 (minimum), 1 (maximum), and 0.5 (half point of the range) (Bevilacqua et al., 2010a; Bevilacqua and Sinigaglia, 2010). In this research, the independent variables were the concentration of the flour (F), the inoculum of *L. acidophilus* (I), and the incubation temperature (T). More specifically, a design was developed using GFF (combinations A-F, design named GFA) and a second one using CF (combinations G-N, design named CFA).

Table 1 reports the 12 combinations of the centroids and two controls (CNT-1 and CNT-2), i.e., two further combinations where no flour was added.

TABLE 1 | Acidification of *L. acidophilus* La-5: fitting parameters of the lag-exponential equation (mean and SE).

Combination	Coded levels			Values			ΔpH_{max}	d_{max}	α	R
	I	F	T	I	F	T				
GFA										
A	1	0	0	8	0	30	2.73 ± 0.08	0.22 ± 0.01	–	0.978
B	0	1	0	4	5	30	1.68 ± 0.08	0.11 ± 0.01	2.00 ± 2.45	0.977
C	0	0	1	4	0	45	2.47 ± 0.11	0.14 ± 0.01	1.10 ± 2.63	0.978
D	0.5	0.5	0	6	2.5	30	2.59 ± 0.07	0.14 ± 0.01	1.89 ± 1.75	0.990
E	0.5	0	0.5	6	0	37.5	3.14 ± 0.03	0.24 ± 0.00	–	0.998
F	0	0.5	0.5	4	2.5	37.5	2.97 ± 0.10	0.21 ± 0.01	3.09 ± 2.04	0.982
CFA										
G	1	0	0	8	0	30	2.76 ± 0.07	0.20 ± 0.01	–	0.986
H	0	1	0	4	5	30	2.28 ± 0.09	0.12 ± 0.01	2.74 ± 2.31	0.984
I	0	0	1	4	0	45	2.58 ± 0.10	0.13 ± 0.01	1.21 ± 2.41	0.983
L	0.5	0.5	0	6	2.5	30	2.51 ± 0.08	0.14 ± 0.01	1.26 ± 2.03	0.987
M	0.5	0	0.5	6	0	37.5	2.98 ± 0.03	0.25 ± 0.00	–	0.998
N	0	0.5	0.5	4	2.5	37.5	2.94 ± 0.09	0.21 ± 0.01	2.68 ± 2.02	0.983
CNT-1	–	–	–	8	0	45	2.44 ± 0.08	0.21 ± 0.03	–	0.967
CNT-2	–	–	–	6	0	45	2.44 ± 0.07	0.17 ± 0.01	–	0.985

I, inoculum (log cfu/ml); F, flour (g/l); and T, temperature, (°C).
GFA, design with gluten friendly; CFA, design with control flour.
ΔpH_{max}, acidification (decrease of pH at the end of the assay); d_{max}, acidification rate (ΔpH/day); α, time before the beginning of acidification (h); R, regression coefficient.

Samples Preparation

According to the design, *L. acidophilus* was inoculated to 4 – 6 – 8 log cfu/ml in 15 ml of pasteurized milk supplemented with variable amounts of GFF or CF (0.0 – 2.5 – 5.0 g/l); then, the samples were incubated at 30, 37.5 or 45°C for 72h. The analyses were done after 4, 6, 15, 18, 21, 24, 28, 30, 39, 48, and 72h of incubation.

The acidification was monitored through pH measuring by a pH-meter (Crison, Barcelona, Spain).

Modeling

The experiments were performed in duplicate over two different samples; for each batch the measurements were repeated twice.

The data were modeled as acidification (ΔpH), i.e., pH decrease referred to the beginning of the experiment. ΔpH was used as the dependent variable for a primary modeling through the lag-exponential model by van Gerwen and Zwietering (1998) and by Baty and Delignette-Muller (2004), cast in the following form:

$$\Delta pH = \begin{cases} 0 & t \leq \alpha \\ \Delta pH_{max} - \log\{1 + (10^{\Delta pH_{max}} - 1) * \exp[-d_{max}(t - \alpha)]\} & t > \alpha \end{cases}$$

where: ΔpH and t are the dependent and independent variables, respectively (acidification and time-h); α is the time before the beginning of the acidification kinetic (h); d_{max} is the maximal acidification rate (1/h); ΔpH_{max} is the maximum level of acidification.

When the acidification kinetic did not show the parameter α, the lag-exponential model was used as follows (Delignette-Muller et al., 2006):

$$\Delta pH = \Delta pH_{max} - \log \{1 + (10^{\Delta pHmax} - 1) * \exp(-d_{max}t)\}$$

In a second step, ΔpH_{max} and d_{max} were used as input values for a multiple regression approach; the temperature, the level of inoculum and the amount of flour were used as independent variables. The analysis was done through the software Statistica for Windows (StatSoft, Tulsa, OK, United States), option Design of Experiments/mixture designs.

The model was built by using the option "quadratic," for the evaluation of the individual ("Flour," "Inoculum," and "Temperature") and interactive effects ("Flour ∗Inoculum," "Flour∗Temperature," and "Temperature∗Inoculum").

The most important output of the modeling was a polynomial equation reading as follows:

$$y = B_0 + \Sigma B_i x_i + \Sigma B_{ij} x_i x_j$$

where, y and x_i and x_j are respectively, the dependent and the independent variables; B_i, and B_{ij} are the coefficients of the model. This model assessed the effects of linear (x_i), and interactive terms ($\Sigma x_i x_j$) of the independent variables on the dependent variable.

The significance of the model was evaluated through the adjusted regression coefficients and the mean square residual, whereas the significance of each factor was assessed through the Fisher test ($P < 0.05$). A second output of the polynomial equation is the ternary plots.

The effect of each independent variable (inoculum, temperature, flour) on the fitting parameters of the acidification kinetic of *L. acidophilus* (ΔpH_{max} and d_{max}) was evaluated through the individual desirability functions, estimated as follows:

$$d = \begin{cases} 0, & y \leq y_{min} \\ (y - y_{min})/(y_{max} - y_{min}) & y_{min} \leq y \leq y_{max} \\ 1, & y \geq y_{max} \end{cases}$$

Where y_{min} and y_{max} are the minimum and maximum values of the dependent variable, respectively. The desirability was included in the range 0–1 (0 for the lowest value of ΔpH_{max} and d_{max} and 1 for their maximal values). The desirability profiles were built by setting the variables to the coded level 0.33 (inoculum to 5.3 log cfu/ml, temperature to 35°C, and flour to1.65g/l).

Product Realization

Samples Preparation

Three different productions of functional fermented milk were realized as follows: one batch added with GFF (2.5 g/l) (GFA), another batch added with CF (2.5 g/l) (CFA) and a control batch without flour (LA). More specifically, fresh whole pasteurized homogenized cow's milk was added with flours, inoculated with *L. acidophilus* at 6.5 log cfu/ml and left to ferment at 37°C for 2 days. The fermentation was monitored by measuring the pH through a pH electrode 50*50T CRISON (Crison Instruments, Barcelona, Spain). After the fermentation, the samples were stored at 4°C for 90 days; microbiological and sensorial analyses, measurements of pH, a_w and color were made as detailed below.

For microbiological analyses the following media were used: MRS Agar (MRSA) acidified to pH 5, incubated at 37°C under anaerobiosis for *L. acidophilus*; MRSA incubated at 30 and 42°C for 48h under anaerobiosis, for mesophilic and thermophilic lactobacilli, respectively; M17 incubated at 30 and 42°C for 48h under anaerobiosis, for lactococci and streptococci, respectively; Slanetz/Bartley Agar incubated at 37°C for 48h, for enterococci; Plate Count Agar (PCA) incubated at 5°C for a week or 32°C for 48h for psychrotrophic bacteria and mesophilic bacteria, respectively; Baird-Parker agar base, with egg yolk tellurite emulsion, incubated at 37°C for 48 h for staphylococci and *Micrococcaceae*; Pseudomonas Agar Base (PAB) with CFC Selective Supplement incubated at 25°C for 48h for *Pseudomonas* spp.; Violet Red Bile Glucose Agar (VRBGA), incubated at 37°C for 24h for *Enterobacteriaceae*; Violet Red Bile Agar (VRBA) incubated at 37°C or 42°C for 18–24h for total and fecal coliforms, respectively; Sabouraud dextrose agar, supplemented with chloramphenicol (0.1 g/l) (C. Erba, Milan, Italy), incubated at 25°C for 48h or 5 days, for yeasts and molds, respectively. All the media and the supplements were from Oxoid.

The viable count of *L.acidophilus* was confirmed by a random isolation of some colonies, microscopic, phenotypic tests and PCR analysis (Speranza et al., 2015).

At each sampling time, pH values were measured in duplicate by a pH-meter and a_w values were measured in triplicate by an AQUALAB CX-2 (Decagon Device, Pullman, WA, United States). Color was evaluated by a colorimeter Chroma Meter (Minolta, Japan) by measuring CIE L^* (lightness), a^* (redness) and b^* (yellowness) values.

The sensory evaluation panel consisted of 15 panelists aging between 22 and 38 years (students and researchers of the Department of the Science of Agriculture, Food and Environment (SAFE), University of Foggia). Using a scale ranging from 0 to10 (where 0 stands for the most attractive attributes and 0 for the absolutely unpleasant attributes), the sensorial overall quality of the samples was determined by evaluating color, odor and overall acceptability.

Modeling

The experiments were repeated twice on two independent samples. The results of *L.acidophilus* were modeled as decrease of viable count over the time and fitted through the lag-exponential model, as reported above for the acidification kinetic.

The sensory scores were analyzed through the non-parametric test of Kruskal-Wallis (Analysis of Variance by ranks); the critical value of P was set to 0.05.

TABLE 2 | Standardized effects for flour, inoculum, and temperature on the maximum acidification (ΔpH_{max}) and acidification rate (d_{max}) of *L. acidophilus* in presence of Gluten Friendly (design GFA) and control flour (design CFA).

	ΔpH_{max}		d_{max}	
	GFA	**FCA**	**GFA**	**FCA**
Inoculum	57.878	58.610	33.734	35.784
Flour	35.500	48.367	17.393	20.985
Temperature	52.297	54.864	22.194	23.562
Inoculum*flour	6.676	–*	–2.478	–2.864
Inoculum*temperature	9.367	5.472	7.922	11.454
Flour*temperature	15.484	8.805	10.499	11.392
R^2_{ad}	0.972	0.903	0.949	0.952

R^2 a_d, determination coefficient adjusted for the multiple regression.
∗Not significant.

RESULTS

First Phase: Product Optimization

The first step was a product optimization to design a synbiotic active drink, fermented with *L. acidophilus* La-5 and containing GFF as a beneficial ingredient. At this scope, the screening was aimed at choosing the optimal conditions to produce the active drink, in terms of level of inoculum, temperature and amount of flour. Therefore, two different designs were performed: the first with the control flour (design CFA) and the second one with the GFF (design GFA).

A requisite to design a synbiotic food is that the prebiotic component/beneficial ingredient must not affect the performances of the starter and/or probiotic microorganisms; thus, the acidification of *L. acidophilus* was assessed in presence of flour. **Table 1** shows the combinations of the design and the performances of the probiotic as ΔpH_{max} (maximum acidification) and d_{max} (acidification rate). In the design with GFF, ΔpH varied from 1.68 to 3.14 and the acidification rate from 0.11 to 0.24h^{-1}. Similar results were found for the CF (acidification in the range 2.28 – 2.98 and rate 0.12 – 0.25h^{-1}).

As an example, **Figure 1** shows the kinetic of acidification in two selected combinations of the design.

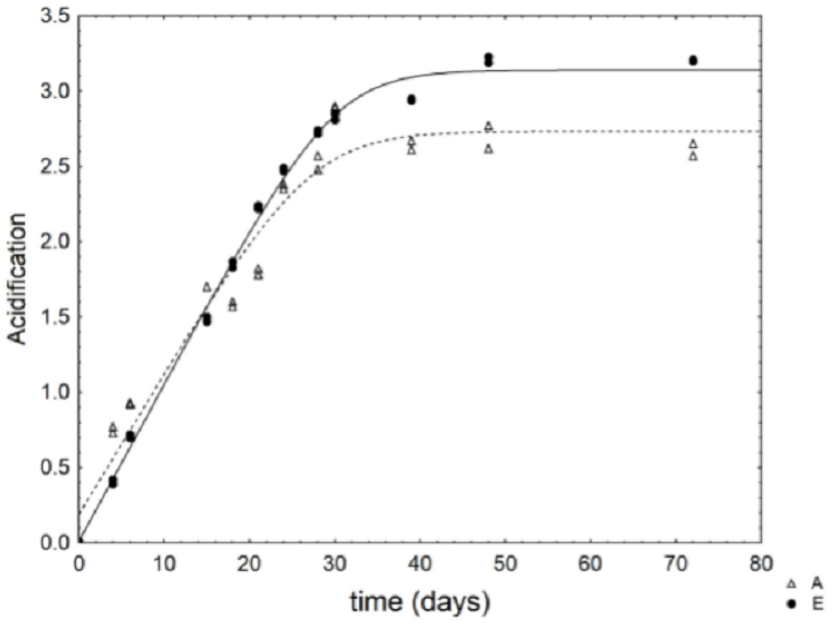

FIGURE 1 | Kinetic of acidification of *L. acidophilus* in some selected combinations of the mixture design (**Table 1**). The lines represent the best fit through the lag-exponential model.

In the second step of the screening, ΔpH_{max} and d_{max} were used as dependent variables for a multiple regression approach. The first output was the table of the standardized effects, showing the statistical

weight and the significance of each individual (flour, inoculum, and temperature) and interactive factors. All variables were significant as individual terms on both ΔpH_{max} and d_{max}; however, the most significant factor was the level of inoculum, followed by the temperature and finally by the amount of flour.

ΔpH_{max} and d_{max} were also affected by the interactive terms and generally the strongest weight was found for the interaction "flour∗temperature" (**Table2**).

Other outputs of a mixture design are the ternary plots, showing the interactions of three factors in a bi-dimensional space. For the sample GFA (containing GFF), the model predicted the maximum extent of acidification at the coded level 0.5 of inoculum and temperature (6 log cfu/ml and 37°C) and with an amount of flour in the range 0.0 – 0.50 (from 0 to 2.50 g/l) (**Figure 2A**). Similar results were found for the acidification in presence of the control flour (CFA) (**Figure 2B**), although the effect of flour seemed stronger and the acidification was lower at the coded level 0.25 (1.25g/l).

Figure 3 shows the results for the acidification rate. The model predicted the highest values of this parameter when both inoculum and temperature were at the coded levels 0.5.

A ternary plot is an important tool; however, it could not be used to analyze the quantitative effect of each individual term. A solution to counteract this limit is the use of the desirability approach.

The desirability is a dimensionless parameter, ranging from 0 to 1 and is the answer to question: how much desired is an output? The reply is: 0 for the worst result (the lowest values of acidification and acidification rate) and 1 for the best one (the highest values of acidification and acidification rate). Moreover, a desirability profile is often completed by a prediction profile, which shows the predicted values of the dependent variable as a function of the coded values of the factors of the design.

Figure 4 shows the desirability profiles for ΔpH_{max} in presence of GFF; the effect of the level of inoculum was not strictly linear but quadratic. An increase of the level of inoculum, in fact, could exert a negative effect of the extent of acidification with a decreased performance of *L.acidophilus* at the coded level 1 (inoculum at 8 log cfu/ml). A quadratic effect was also found for the temperature, with a decreased effect at the coded levels 0 and1(30 and 45°C).

The correlation "flour vs. acidification" was negative; the model predicted the highest value of acidification for the coded levels 0 and 0.25 (0 and 1.25g/l) and a level of acidification nearby the optimal value (ca. 3) at the coded level 0.5 (2.5 g/l of GFF); on the other hand, the extent of acidification strongly decreased at the coded levels 0.75 and 1.0, thus suggesting a negative effect of the flour on the performances of the probiotic.

The desirability profiles showed similar trends in presence of the control flour (**Figure 5**), with a negative effect on the acidification for an inoculum of 8.0 log cfu/ml, an amount of flour >2.5g/l and a temperature at 30 and 45°C.

The desirability profiles of d_{max} for both GFF and CF suggested the same results (data not shown). The outputs of the desirability and prediction profiles were used to design/optimize the conditions to produce the active drink; thus, the variables for the fermentation of milk were set asfollows:

• *L.acidophilus* at 6.5 log cfu/ml
• Flour at 2.5 g/l
• Temperature at 37° C.

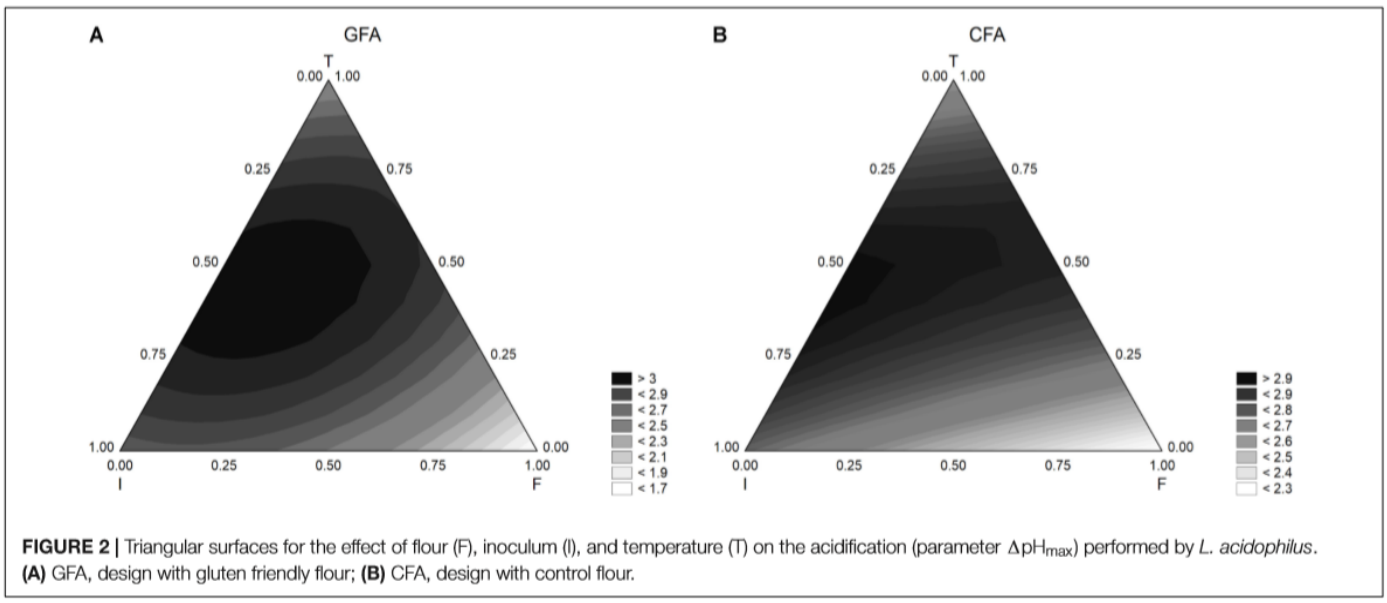

FIGURE 2 | Triangular surfaces for the effect of flour (F), inoculum (I), and temperature (T) on the acidification (parameter ΔpH_{max}) performed by *L. acidophilus*. **(A)** GFA, design with gluten friendly flour; **(B)** CFA, design with control flour.

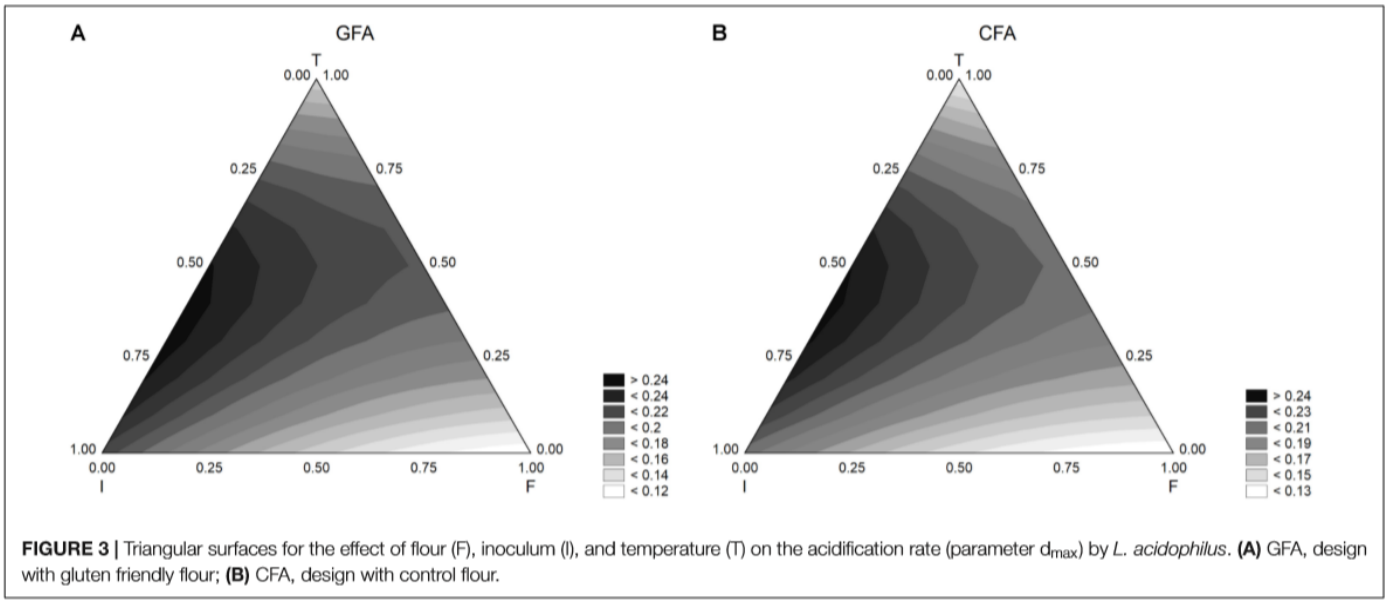

FIGURE 3 | Triangular surfaces for the effect of flour (F), inoculum (I), and temperature (T) on the acidification rate (parameter d_{max}) by *L. acidophilus*. **(A)** GFA, design with gluten friendly flour; **(B)** CFA, design with control flour.

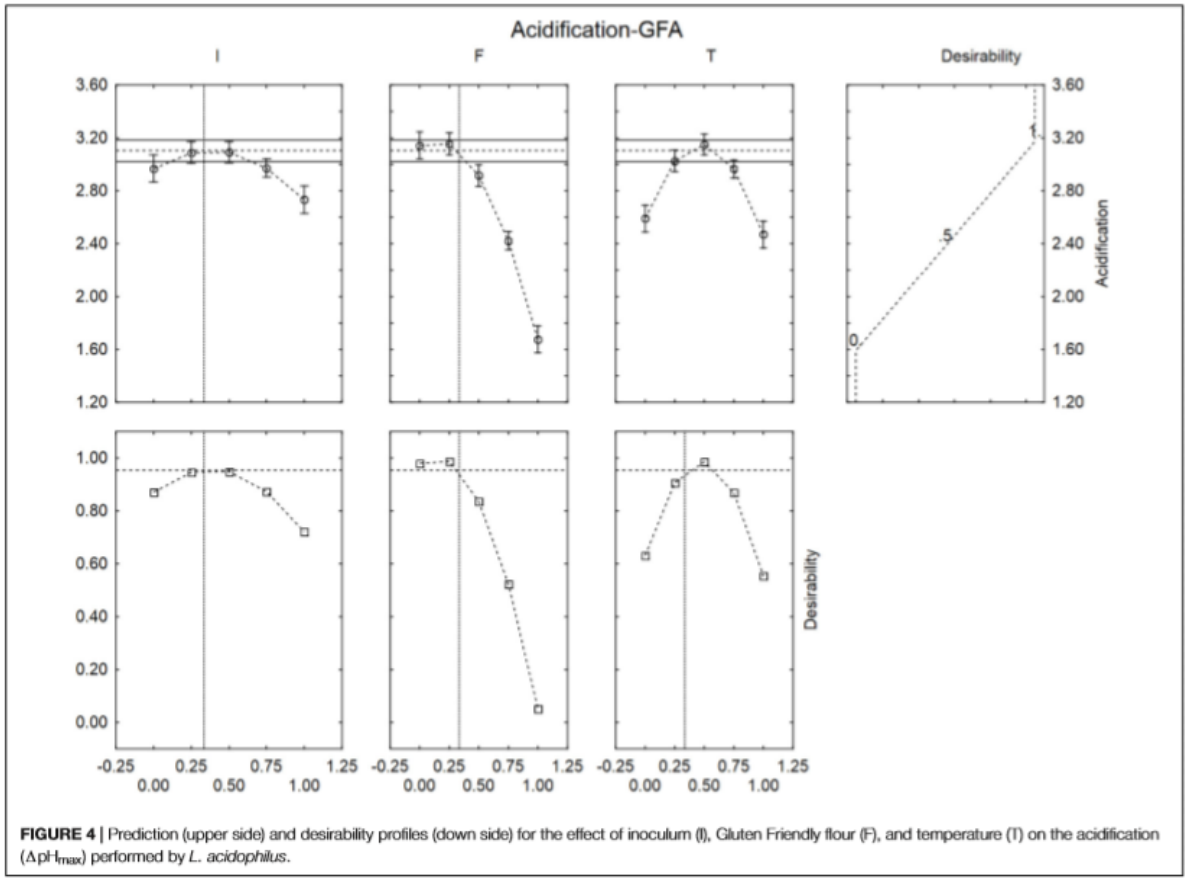

FIGURE 4 | Prediction (upper side) and desirability profiles (down side) for the effect of inoculum (I), Gluten Friendly flour (F), and temperature (T) on the acidification (ΔpH_{max}) performed by *L. acidophilus*.

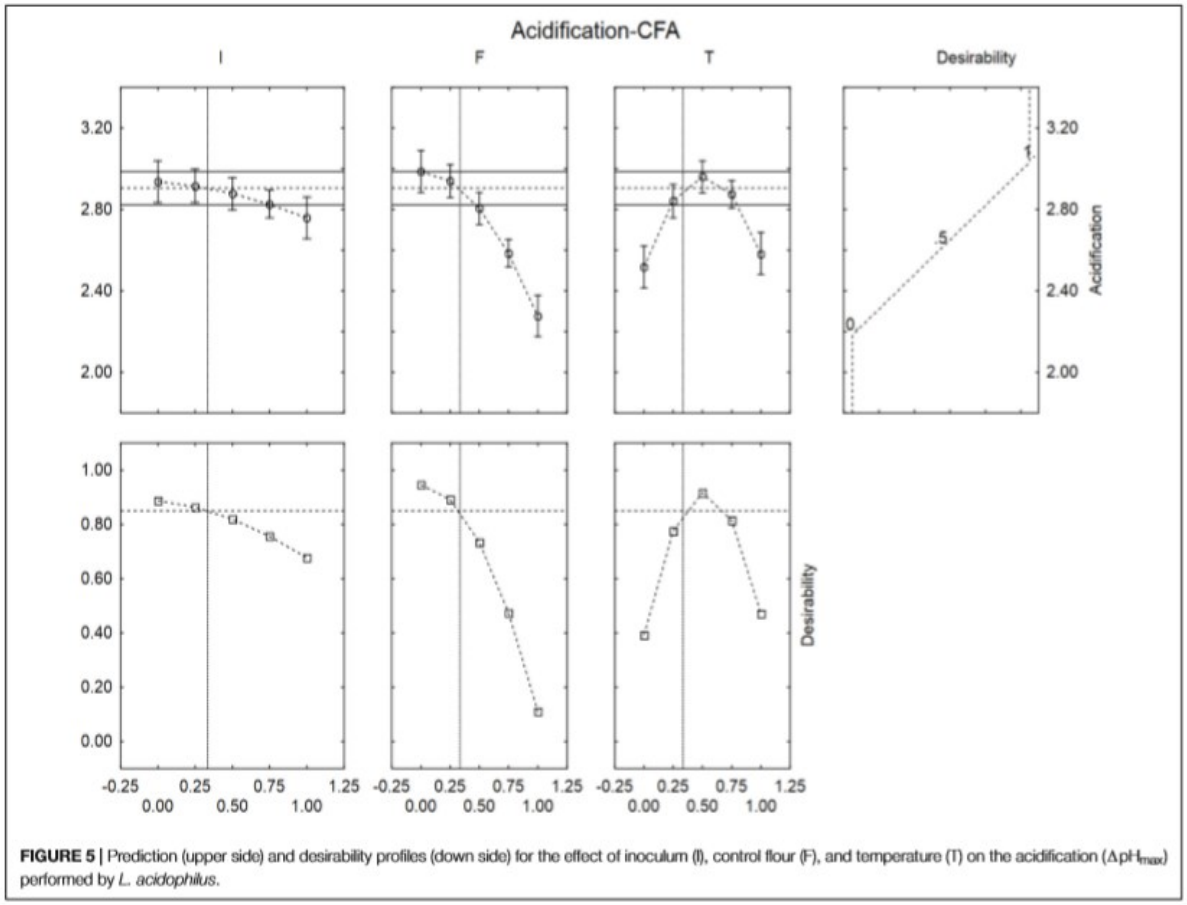

FIGURE 5 | Prediction (upper side) and desirability profiles (down side) for the effect of inoculum (I), control flour (F), and temperature (T) on the acidification (ΔpH_{max}) performed by *L. acidophilus*.

Second Phase: Product Realization

After the fermentation the viable count of probiotic was 8.30 log cfu/ml, the pH 4.11 (LA) and 4.0 (CFA and GFA) and the Aw 0.990.

Figure 6 shows the results for the viable count of *L.acidophilus* throughout the refrigerated storage of the synbiotic drink. The probiotic never attained the critical level (7 log cfu/ml) (Rosburg et al., 2010) (data not shown); however, the supplementation of GFF exerted a significant effect on the shape of the death kinetic. The probiotic experienced a shoulder length (SL), i.e., a time before the beginning of the exponential death kinetic; this parameter was significantly affected by the formulation. The probiotic alone (sample LA) experienced a SL of 11.25 days and the addition of control flour did not act on it (FCA, 13.44 days) ($P > 0.05$). On the other hand, the supplementation of GFF significantly prolonged SL and increased it to 20.35 days (sample GFA) ($P < 0.05$).

Concerning the other microbiological results, the levels of enterobacteria, pseudomonads, yeasts and molds, enterococci and psychrotrophic bacteria were always below the detection limit (data not shown). Both pH and a_w did not undergo significant changes throughout storage (data not shown).

Figure 7 shows the results for the instrumental color evaluation (luminosity, L). A significant decrease of L was found after 56 days and this trend was more pronounced in the sample CFA, containing the control flour.

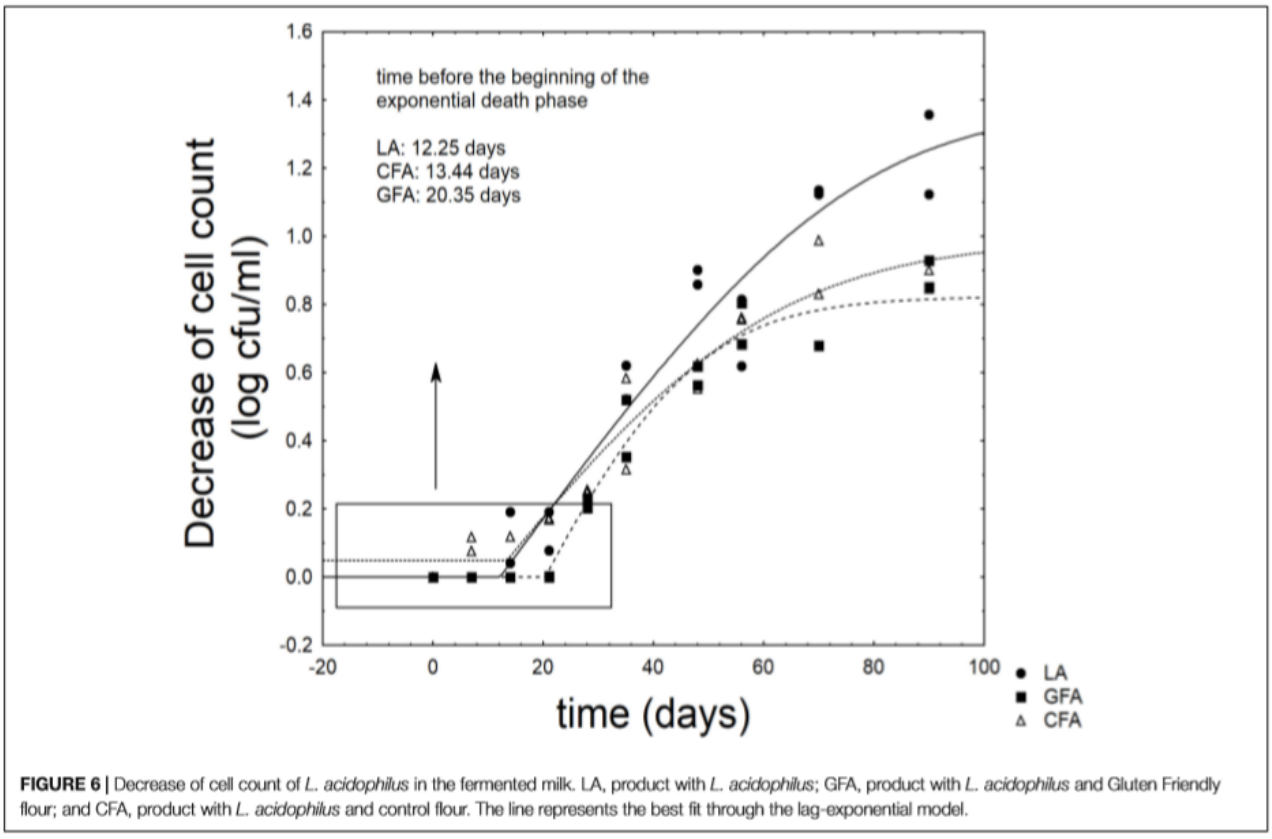

FIGURE 6 | Decrease of cell count of *L. acidophilus* in the fermented milk. LA, product with *L. acidophilus*; GFA, product with *L. acidophilus* and Gluten Friendly flour; and CFA, product with *L. acidophilus* and control flour. The line represents the best fit through the lag-exponential model.

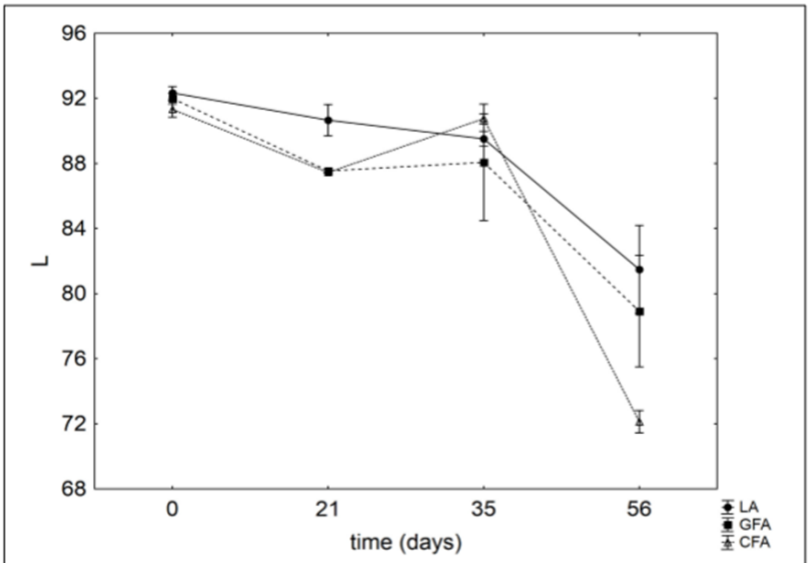

FIGURE 7 | Evolution of L (mean values ± SE). LA, product with *L. acidophilus*; GFA, product with *L. acidophilus* and Gluten Friendly flour; and CFA, product with *L. acidophilus* and control flour. The numbers after the acronym of the samples indicate the days of storage (analysis after 7, 14, 21, 28, 35, and 56 days).

Concerning the sensory scores, **Figure 8** shows the results for the overall quality; the results were analyzed through a nonparametric test, as they did not fit with the basic requisite of a parametric statistic (normal distribution). The median score was higher than the break-point (score, 5) for 28 days; then it decreased. However, the differences amongst the samples were not significant.

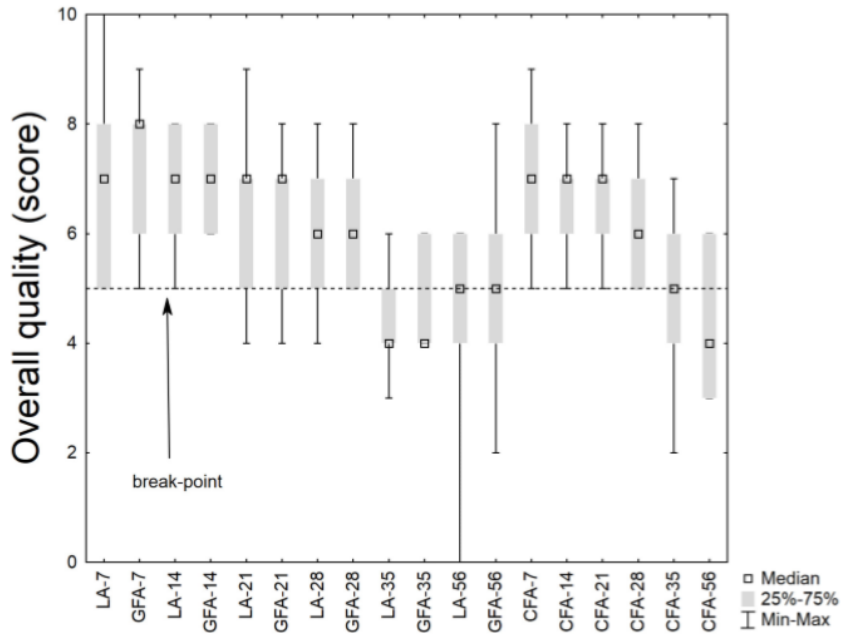

FIGURE 8 | Box-whisker plot on the sensory score (overall quality). LA, product with *L. acidophilus*; GFA, product with *L. acidophilus* and Gluten Friendly flour; and CFA, product with *L. acidophilus* and control flour. The numbers after the acronym of the samples indicate the days of storage (analysis after 7, 14, 21, 28, 35, and 56 days).

DISCUSSION

The starting point of this research was a patent about the use of a novel temperature-based method on wheat kernels to induce structural changes in gluten proteins (Gluten Friendly™) (Lamacchia et al., 2013, 2015). Some investigations performed on this approach also focused on the effect of the bread produced with GFF on some probiotic and foodborne strains (*L. acidophilus*, *Bifidobacterium animalis* subsp. *lactis, Staphylococcus aureus*, and *Salmonella Typhimurium*) to pinpoint a potential modification of the survival of these selected targets under strict controlled conditions: in particular, the study was conducted to determine whether GFF could have a beneficial effect by modifying the qualitative-quantitative composition of gut microbiota (Bevilacqua et al., 2016b). The results of the mentioned study highlighted a protective effect on *L. acidophilus* viability observing a significant lowering of its death rate and a prolongation of the survival time from 70.28 to 93.46 h with 0.8 g/l of GFF bread; therefore, the idea to design a synbiotic fermented milk containing GFF as a beneficial ingredient and *L. acidophilus* as functional starter was exploited in this experimentation. During the design of a synbiotic food, it is mainly important that the prebiotic component must not affect the performances of the starter and/or probiotic microorganisms; thus, the first phase of this research was performed to assess the acidification of *L. acidophilus* in presence of flour and as a function of the level of inoculum and temperature to optimize the conditions to produce the active drink. Since a negative effect on the performances of the strain was observed for an inoculum of 8.0 log cfu/ml, an amount of flour >2.5 g/l and a temperature at 30 and 45°C, the fermented milk was produced by inoculating *L. acidophilus* at 6.5 log cfu/ml, adding flour at 2.5 g/l and performing the fermentation at 37°C. During the refrigerated storage of the synbiotic drink produced, the obtained results confirmed those obtained by Bevilacqua et al. (2016b); in particular, the positive effect of GFF on the death kinetic of the probiotic was evidenced by a significant prolongation of the shoulder length of the test microorganism (to 20.35 days), thus confirming the lowering of the death rate as previously reported. As reported in literature, prebiotics could act toward lactobacilli through three possible ways: effect on metabolism, induction of a higher stability of membrane and starvation. For example, fructooligosaccharides (FOSs) are reported to affect the regulation of genes involved in the primary metabolism (fructokinase, phosphoenolpyruvate transport system, β-fructofuranosidase, and α-glucosidase) and/or on the regulation of genes linked to the synthesis of fatty acids, proteins and cell wall (Saulnier et al., 2007). On the other hand, some studies reported a protective effect of fructans (inulin and FOS) by inducing a higher stability of membrane against some stresses (freezing, dehydration, etc.) (Vereykenetal., 2001, 2003a,b), due to a probable interaction with the phospholipids of the membrane (Schwab et al., 2007). The third way of action was observed by Altieri et al. (2011) which suggested the induction of a kind of starvation when *L. plantarum* was grown in

a medium containing inulin or FOS; this effect was also suggested by Saulnier et al. (2007), Hussain et al. (2009), Bevilacqua et al. (2010b, 2016a, c) and by Wang et al. (2011) which observed an increase in viability as practical output of starvation.

The data collected on GFF suggest that it cannot be simply labeled as a prebiotic (Bevilacqua et al., 2016b; Lamacchia et al., 2018).The protective effect observed for GFF could be considered quite different from those exerted by some prebiotics, because it probably did not induce resistance in cells but lowered the death rate. This effect was previously observed in some cell-free filtrates or bifidogenic factors which enhanced growth by altering membrane permeability, combating cell aging, etc. (Oda et al., 2013; Kang et al., 2015). These bifidogenic factors are different from the traditional prebiotics because of their nature (peptides orproteins).

A way to elucidate and explain the effect of GFF is in its technology. As supposed elsewhere (Bevilacqua et al., 2016b; Lamacchia et al., 2016), the alternation of high temperatures to evaporation phases induces different spatial conformation of the amino acid sequences in hydrated wheat caryopses treated with the Gluten Friendly™ technology, inducing a rearrangement of the secondary and tertiary structure of the gluten proteins.

This rearrangement was suggested as the main cause for an exposure of the positive charge, which in turn could be responsible of an interaction with the teichoic acids of the cell wall of lactobacilli. This interaction could lead to a protection of the cell from aging, and/or a change in the membrane permeability (Bevilacqua et al., 2016b). This effect was later found and recovered on the lactobacilli of the fecal microbiota (Costabile et al., 2017).

The idea of positive charges was indirectly confirmed by the different bioactivity toward lactobacilli and *Salmonella* sp., a Gram negative (Bevilacqua et al., 2016b). *Salmonella* possesses the outer membrane, and different distribution of charges. Moreover, the distribution of charges, probably associated with teichoic acids, could explain the quick effect on lactobacilli and the delayed effect on bifidobacterial.

Another possible idea, suggested by some preliminary evidences, is a possible use of the modified gluten as an alternative source of nutrients by lactobacilli under stressful conditions; however, this hypothesis should be confirmed by ad hoc investigations. Maybe, the mode of action is in the middle with a combination of a positive effect on permeability and the use as nutrient.

An important requisite in the design of a synbiotic food is that the probiotic concentration at the time of sale was higher than a break-point fixed in 106 cfu/g or cfu/ml by the Italian legislation (Fortina, 2007) and recently increased to 107 cfu/g or 109 per day (Rosburg et al., 2010; Italian Ministry of Health, 2018). Our results showed that the population of *L. acidophilus* throughout the refrigerated storage of the synbiotic drink never attained the critical level (7 log cfu/ml) and no adverse effects on

organoleptic characteristics of the product were exerted by both the probiotic organism and the prebiotic addition.

The novelty of this paper is a structured statistical approach on the quantitative effects of GFF on the survival of *L.acidophilus* La-5 and represents the first step to setup and design a synbiotic fermented milk, combining this probiotic and GFF. The practical implication of the results could be summarized as follows:

(a) The flour could be used at a maximum concentration of 2.5 g/l otherwise it could negatively affect the acidification kinetic.

(b) The supplementation of GFF exerted a positive effect on the viability of the probiotic, with a prolongation of the shoulder length to 20 days, whereas in the control or in presence of CF the shoulder length was ca.12 – 13 days.

(c) The use of GFF did not act on the sensory scores and on the physico-chemical parameters.

(d) The idea of a functional product combining a probiotic and GFF is technologically feasible.

The benefits and the added value of this new product is the possibility of using an ingredient able to exert a dual effect: (i) to act in the product by increasing the viability of the probiotic (economic value and prolonged shelf life) (results of this paper); (ii) to positively modulate the microbiota of celiac and healthy people (functional effect) (results of the previous researches).

Some evidences suggested that the increased survival of *L.acidophilus* could be the result of a shift of the death curve with a prolonged shoulder length; however, further experiments are required to try to elucidate the molecular mechanisms beyond it (change in the permeability of the membrane, possible use of the modified gluten as an alternative nutrient source or other mechanisms).

In conclusion, this research aimed at designing an approach and a method to combine GF with probiotics in fermented milk and *L. acidophilus* La-5 was used as the test microorganism; the results of this research showed the suitability of this method and suggested the possibility of using it for many other strains.

AUTHOR CONTRIBUTIONS

MC, MS, and CL conceived the study. AB, BS, DC, and MC designed the experiments. CL and DM prepared the GFF. BS, DC, and DM performed the experiments. AB performed the statistic. BS and AB wrote the manuscript. All authors interpreted the results and reviewed the paper. CL funded the research.

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REFERENCES

Akin, M. B., Akin, M. S., and Kimarci, Z. (2007). Effects of inulin and sugar levels on the viability of yogurt and probiotic bacteria and the physical and sensory characteristics in probiotic ice-cream. Food Chem. 104, 93–99. doi: 10.1016/j.foodchem. 2006.11.030

Al-Sheraji, S. H., Ismail, A., Manap, M. Y., Mustafa, S., Yusof, R. M., and Hassan, F. A. (2013). Prebiotics as functional foods: a review. J. Funct. Foods 5, 1542– 1553. doi: 10.1016/j.jff.2013.08.009

Altieri, C., Bevilacqua, A., and Sinigaglia, M. (2011). Prolonging the viability of *Lactobacillus plantarum* through the addition of prebiotics into the medium. J.Food.Sci.76, M336–M345. doi: 10.1111/j.1750-3841.2011.02269.x

Bafna, H. P., Ajithkrishnan, C. G., Kalantharakath, T., Singh, R. P., Kalyan, P., Vathar, J. B., et al. (2018). Effect of short-term consumption of amul probiotic yogurt containing *Lactobacillus acidophilusand Bifidobacterium lactis* Bb12 on salivary *Streptococcus mutants* counts in high caries risk individuals Int. J. Appl. BasicMed.Res.8,111–115. doi: 10.4103/ijabmr.IJABMR_447_16

Baty, F., and Delignette-Muller, M. L. (2004). Estimating the bacterial lag time: whichmodel, which precision? Int.J.Food Microbiol.91,261–277. doi: 10.1016/ j.ijfoodmicro.2003.07.002

Bevilacqua, A., Corbo, M. R., and Sinigaglia, M. (2010a). "Design of experiments: a powerful tool in food microbiology," in Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, ed. A. Mendez-Vilas (Badajoz: Formatex Research Center), 1419–1429.

Bevilacqua, A., Corbo, M. R., Sinigaglia, M., Speranza, B., Campaniello, D., and Altieri, C. (2016a). Effects of inulin, fructooligosaccharides/glucose and pH on the shape of the death kinetic of *Lactobacillus reuteri* DSM 20016. Int. J. Food Sci.Tech.51,2251–2259. doi: 10.1111/ijfs.13205

Bevilacqua, A., Costabile, A., Bergillos-Meca, T., Gonzalez, I., Landriscina, L., Ciuffreda, E., et al.(2016b). ImpactofGluten-Friendly bread on the metabolism and function of in vitro gut microbiota in healthy human and coeliac subjects. PLoSOne11:0162770. doi: 10.1371/journal.pone.0162770

Bevilacqua, A., and Sinigaglia, M. (2010). "Food shelf life and safety: challenge tests, prediction and mathematical tools," in Application of Alternative Food Preservation Technologies to Enhance Food Safety and Stability, eds A. Bevilacqua, M. R. Corbo, and M. Sinigaglia (United Arab Emirates: Bentham Publisher),161–187. doi: 10.2174/978160805096311001010161

Bevilacqua, A., Sinigaglia, M., and Corbo, M. R. (2010b). An acid/alkaline stress and the addition of amino acid induce a prolonged viability of *Lactobacillus plantarum* loaded into alginate gels. Int. J. Food Microbiol. 142, 242–246. doi: 10.1016/j.ijfoodmicro.2010.05.030

Bevilacqua, A., Sinigaglia, M., Speranza, B., and Altieri, C. (2016c). Effect of prebiotic compounds on the growth and survival of bifidobacteria in a laboratory medium. Adv. J. Food Sci. Tech. 11, 770–774. doi: 10.19026/ajfst.11. 2790

Brasili, E., Mengheri, E., Tomassini, A., Capuani, G., Roselli, M., FInamore, A., e tal. (2013). *Lactobacillus acidophilus* La-5 and *Bifidobacterium lactis* Bb12 induce different age-related metabolic profiles revealed by 1H-NMR spectroscopy in urineandfecesofmice.J.Nutr.143,1549–1557. doi: 10.3945/jn.113.177105

Cencic, A., and Chingwaru, W. (2010). The role of functional foods, nutraceuticals, and food supplements in intestinal health. Nutrients 2, 611–625. doi: 10.3390/ nu2060611

Costabile, A., Bergillos-Meca, T., Landriscina, L., Bevilacqua, A., GonzalezSalvador, I., Corbo, M. R., et al. (2017). An in vitro fermentation study on the effects of Gluten Friendly bread on microbiota and short chain fatty acids of fecal samples from healthy and celiac subjects. Front. Microbiol. 8:1722. doi: 10.3389/fmicb.2017.01722

Cruz, A. G., Antunes, A. E., Sousa, A. L., Faria, A. F., and Saad, M. I. (2009). Icecream as a probiotic food carrier. Res. Int. Food 42, 1233–1239. doi: 10.1016/j. foodres.2009.03.020

Delignette-Muller, M. L., Cornu, M., Pouillot, R., and Denis, J. B. (2006). Use of the Bayesian modelling in risk assessment: application to growth of *Listeria monocytogenes* and food flora in cold-smoked salmon. Int. J. Food Microbiol. 106,195–208. doi: 10.1016/j.ijfoodmicro.2005.06.021
FAO/WHO (2006). "Probiotic in foods. Health and nutritional properties and guidelinesforevaluation," in FAO Food and Nutrition (Rome: FAO), 85.
Fortina, M. G. (2007). "I prodotti lattiero-caseari," in La Microbiologia Applicata Alle Industrie Alimentari, eds L. S. Cocolin and G. Comi (Roma: Aracne Editrice), 289–336.
Gebara, C., Chaves, K. S., Ribeiro, M. C. E., Souza, F. N., Grosso, C. R. F., and Gigante, M. L. (2013). Viability of *Lactobacillus acidophilus* La5 in pectinwhey protein microparticles during exposure to simulated gastrointestinal conditions. FoodRes.Int. 51, 872–878. doi: 10.1016/j.foodres.2013.02.008
González Fabre, A. L. (2008). Más allá de los nutrimentos. Énfasis Alimentación Latinoamérica 6,8–12.
Gotteland, M. (2010). Alimentos simbióticos.Indualimentos 64, 14–15.
Hussain, M. A., Knight, M.I., and Britz, M.L. (2009). Proteomic analysis of lactose starved *Lactobacillus casei* during stationary growth phase. J. Appl. Microbiol. 106,764–773.doi: 10.1111/j.1365-2672.2008.03961.x
Italian Ministry of Health (2018). Guidelines on Probiotics and Prebiotics.Available at: http://www.salute.gov.it/portale/news/p3_2_1_1_1.jsp?lingua=italiano&menu=notizie&p=dalministero&id=3353
Kang, J. E., Kim, T. J., and Moon, G. S. (2015). A novel *Lactobacillus casei* LP1 producing 1,4-dihydroxy-2-naphthoic acid, a bifidogenic growth stimulator. Prev.Nutr.FoodSci.20,78–81.doi: 10.3746/pnf.2015.20.1.78
Lamacchia, C., Di Luccia, A., and Gianfrani, C. (2013). Metodo per la detossificazione delle proteine del glutine dalle granaglie dei cereali. Italian patent n.0001414717. Foggia: Università di Foggia.
Lamacchia, C., Di Luccia, A., and Gianfrani, C. (2015). Method for the detoxification of gluten proteins from grains of cereals. Patent Cooperation Treaty PCT/IB2013/000797. Foggia: University of Foggia.
Lamacchia, C., Landriscina, L., and D'Agnello, P. (2016). Changes in wheat kernels proteins induced by microwave treatment. Food Chem. 197, 634–640. doi: 10. 1016/j.foodchem.2015.11.016
Lamacchia, C., Musaico, D., Henderson, M. E., Bergillos-Meca, T., Roul, M., Landriscina, L., et al. (2018). Temperature-treated gluten proteins in GlutenFriendly™ bread increase mucus production and gut-barrier function in human intestinal goblet cells. J.Funct.Foods 48, 507–514.doi: 10.1016/j.jff.2018. 07.047
Medellin-Peña, M. J., and Griffiths, M. W. (2009). Effect of molecules secreted by *Lactobacillus acidophilus* strain La-5 on *Escherichia coli* O157:H7colonization. Appl.Environ.Microbiol. 75, 1165–1172. doi: 10.1128/AEM.01651-08
Oda, H., Wakabayashi, H., Yamauchi, K., Sato, T., Xiao, J. Z., Abe, F., et al. (2013). Isolation of bifidogenic peptide from the pepsin hydrolysate of bovine lactoferrin. Appl.Environ.Microbiol.79, 1843–1849.doi: 10.1128/AEM. 03343-12
Pokusaeva, K., Fitzgerald, G. F., and van Sinderen, D. (2011). Carbohydrate metabolismin *Bifidobacteria*.Gen.Nutr. 6, 285–306.doi: 10.1007/s12263-0100206-6
Ranadheera, C. S., Evans, C. A., Adams, M. C., and Baines, S. K. (2015). Microencapsulation of *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Propionibacterium jensenii* 702 by spray drying in goat's milk. Small Rum. Res. 123, 155–159. doi: 10.1016/j.smallrumres.2014.10.012
Rosburg, V., Boylston, T., andWhite, P.(2010). Viability of bifidobacteria strains in yogurt with added oat beta-glucan and corn starch during cold storage.J.Food Sci.75, C439–C444.doi: 10.1111/j.1750-3841.2010.01620.x
Saulnier, D., Spinler, J. K., Gibson, G. R., and Versalovic, J. (2009). Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods. Curr. Opin.Biotech.20, 135–141. doi: 10.1016/j.copbio.2009.01.002
Saulnier, D. M. A., Molenaar, D., de Vos, W. M., Gibson, G. R., and Kolida, S. (2007). Identification of prebiotic fructooligosaccharide metabolism in *Lactobacillus plantarum* WCFS1 through microarrays. Appl. Environ. Microbiol.73,1753–1765. doi: 10.1128/AEM.01151-06
Schwab, C., Vogel, R., and Ganzle, M. G. (2007). In? uence of oligosaccharides on the viability and membrane properties of *Lactobacillus reuteri* TMW1.106 during freeze-drying. Cryobiology 55, 108–114. doi: 10.1016/j.cryobiol.2007. 06.004
Senanayake, S. A., Fernando, S., Bamunuarachchi, A., and Arsekularatne, M. (2013). Application of *Lactobacillus acidophilus* (LA5) strain in fruit-based ice cream. FoodSci.Nutr.1, 428–431.doi: 10.1002/fsn3.66

Silva, P. D. L., Bezerra, M. F., Santos, K. M. O., and Correia, R. T. P. (2015). Potentially probiotic ice cream from goat's milk: characterization and cell viability during processing, storage and simulated gastrointestinal conditions. LWT FoodSci.Tech. 62, 452–457. doi: 10.1016/j.lwt.2014.02.055.
Speranza, B., Bevilacqua, A., Corbo, M. R., Altieri, C., and Sinigaglia, M. (2015). Selection of autochthonous strains as promising starter cultures for Fior di Latte cheese, a traditional cheese of Southern Italy. J.Sci.FoodAgric. 95, 88–97. doi: 10.1002/jsfa.6686
van Gerwen, S. J. C., and Zwietering, M. H. (1998). Growth and inactivation models to be used in quantitative risk assessments. J.FoodProtec. 6, 1541–1549. doi: 10.4315/0362-028X-61.11.1541
Vereyken, I. J., Chupin, V., Demel, R. A., Smeekens, S. C. M., and De Kruijff, B. (2001). Fructans insert between the headgroups of phospholipids. Biochim. Biophys.Acta 1510, 307–320. doi: 10.1016/S0005-2736(00)00363-1
Vereyken, I.J., Chupin, V., Islamov, A., Kuklin, A., Hincha, D.K., and DeKruijff, B. (2003a). The effect of fructan on the phospholipid organization in the dry state. Biophys.J. 85, 3058–3065. doi: 10.1016/S0006-3495(03)74724-9
Vereyken, I. J., van Kuik, J. A., Evers, T. H., Rijken, P. J., and De Kruijff, B. (2003b). Structural requirements of the fructan–lipid interaction. Biophys.J.84, 3147–3154. doi: 10.1016/S0006-3495(03)70039-3
Wang, Y., Delettre, J., Corrieu, G., and Beal, C. (2011). Starvation induces physiological changes that act on the cryotolerance of *Lactobacillus acidophilus* RD758. Biotechnol.Prog. 27, 342–350.doi: 10.1002/btpr.566
Zadernowska, A., Chajecka-Wierzchowska, W., andOgryzek, M.P. (2015). Growth potential of *Yersinia enterocolitica* in blue cheese and in blue cheese with probiotic-*Lactobacillus acidophilus* LA-5R.J.FoodSci.Technol. 52, 7540–7544. doi: 10.1007/s13197-015-1873-5
Zarrati, M., Shidfar, F., Nourijelyani, K., Mofid, V., Hossein zadeh-Attar, M. J., Bidad, K., et al. (2013). *Lactobacillus acidophilus* La5, *Bifidobacterium lactis* Bb12, and *Lactobacillus casei* DN001 modulate gene expression of subset of specific transcription factors and cytokines in peripheral blood mononuclear cells of obese and overweight people. Biofactors 39, 633–643. doi: 10.1002/biof.1128

Chapter 4

"Gluten Friendly™"is digested by coeliac subjects and shapes gut microbiota toward homeostasis

(In preparation)

"Gluten Friendly™" is digested by coeliac subjects and shapes gut microbiota toward homeostasis

Angelo Andriulli[1]†, Antonio Bevilacqua[2]†, Orazio Palmieri[1], Anna Latiano[1], Rosanna Fontana[1], Domenica Gioffreda[1], Giuseppe Corritore[1], Ivana Decina[3], Michele Tricarico[3], Daniela Musaico[2], Markku Mäki[4], Jorma Isola[5], Alina Popp[6], Juha Taavela[7], Leonardo Petruzzi[2], Milena Sinigaglia[2], Maria Rosaria Corbo[2]* and Carmen Lamacchia[2]*

correspondence to: carmela.lamacchia@unifg.it and mariarosaria.corbo@unifg.it

1. Introduction

Gluten is a complex mixture of storage proteins in cereals like wheat, barley, and rye. Their high content in proline and glutamine makes them difficult to digest in the gastrointestinal tract (Wieser, 2007). Partial digestion generates peptide sequences that are resistant to further hydrolysis (such as the α-gliadin 33-mer) (van de Wal et al., 1999; Arentz-Hansen et al., 2000; Shan et al., 2002; Molberg et al., 2003; Dewar et al., 2006; Siegel et al., 2006; De Vincenzi et al., 2010; Mamone et al., 2013), and trigger immune responses in celiac and gluten-sensitive patients (Sollid and Khosla, 2005).

Celiac disease (CD) is a chronic intestinal inflammatory disorder due to an aberrant immune response to dietary gluten proteins in genetically predisposed individuals. Gut microbiota variations may play a significant role in the pathogenesis of CD; indeed, dysbiosis is linked with an inflammatory milieu in celiac patients (Marasco et al., 2016). Celiac disease is caused by the interplay between gluten, genetic factors, and environmental factors such as gut microbiota (Nadal et al., 2007). Patients with CD show a reduction in beneficial species (*Lactobacillus* and *Bifidobacterium*) and an increase in those potentially pathogenic (*Bacteroides* and *E. coli*) as compared to healthy subjects (Nadal et al., 2007). The mucus layer is the interface with the microbiota. Exactly, the outer mucus layer is the natural habitat for the commensal bacteria which preserve the function and structure of the gut barrier and regulate the mucosal immune homeostasis (Rao et al., 2013). Several data show that the composition of the mucus layer can affect the microbiota in the gut, whilst the microbiota also determines the properties of the mucus gel (Vancamelbeke et al., 2017). The mucosal layer of CD patients fails to stabilize the gut microbiota and fails to prevent the host from the invasion of harmful antigens and pathogens (Marasco et al., 2016). This dysbiosis is reduced, but may still remain, after a gluten-free diet (De Palma et al., 2010; Marasco et al., 2016).

"Gluten Friendly" is a new type of gluten (Lamacchia et al., 2016) which has shown, *in vitro*, some peculiar characteristics such as: i) a reduced immunoreactivity on gut derived T-cell lines from celiac patients (Lamacchia et al., 2015); ii) a reduced cross-reactivity towards antibodies recognizing the antigenic epitope of gluten proteins (Landriscina et al, 2017). iii) positive modulation of coeliac gut

microbiota (Bevilacqua et al., 2016); iv) bifidogenic effects on gut microbiota of healthy and coeliac subjects (Costabile et al., 2017); v) increase of gut barrier function in human intestinal goblet cells (Lamacchia et al., 2018). We examined the effects of the administration of bread with different doses of "Gluten Friendly", for three months, in coeliac patients treated with a gluten-free diet (GFD) for at least 2 years.

2. Materials and methods

2.1 Therapeutic Intervention Study

A prospective, double-blind, placebo-controlled, randomized trial on either young (15 years old) and adult patients with biopsy-proven Celiac Disease (CD) was carried out at the Division of Gastroenterology, "Casa Sollievo della Sofferenza" Hospital, IRCCS, San Giovanni Rotondo, Italy. The protocol of the study required approval by the Ethics Committee of the Hospital, and the investigation was carried out in compliance with the Good Clinical Practice guidelines of the Helsinki declaration.

Patients

Participants were prospectively recruited from the database of a single tertiary referral hospital, the "Casa Sollievo della Sofferenza" Hospital, located in South of Italy between April and September 2017. Patients were aged 15-75 years, had a biopsy supported CD, and were in remission on a Gluten Free Diet (GFD) for more than 2 years at the time of inclusion. Remission was defined as: (1) negative serology for CD [immunoglobulin A (IgA) anti-tissue transglutaminase 2 (tTG2)] initially screened by the rapid Biocard Celiac test (Anibiotech, Vantaa, Finland) and confirmed by a serum enzyme-linked immunosorbent assay (ELISA) test (Quanta Lite htTG IgA, Inova Diagnostics, Inc. San Diego, CA; 2) negative serology for anti-endomysial antibodies (EMA) at the pre-screening visit (Monkey Endomysium, Bio-Rad, Milan, Italy); (3) on a strictly GFD for at least 24 months; and (4) absence of symptoms which prompted initial diagnosis. After obtaining written informed consent, patients underwent a medical history taking physical examination, laboratory tests and Upper Gastrointestinal Endoscopy (UGE). At screening all subjects were tested for human leukocyte antigen genotype DQ2 and DQ8 using a commercially available Sequence-Specific Oligonucleotide hybridization kit (LABType XR, One Lambda, Canoga Park, CA).

Participants were asked to consume a multi-sugar drink for the permeability test [7.5g lactulose (Duphalac, Solvay Pharmaceuticals Ltd) and 2g D mannitol ≥ 98% (Sigma- Aldrich, UK) in 100 ml of water] (a dosage used widely in oral clinical testing-LAMA testing), in the evening before each study visit at 8 pm and were instructed to collect all overnight and morning first spot pass urine

sample. They also provided a faecal sample for each study visit, for the microbiome analysis and the quantification of gluten in stools.

Consenting patients were also provided with a diary card and asked to provide in, during the study period, information on eventual symptomatic complaints [Celiac Symptom Index Questionnaire (CSI) and Gastrointestinal Symptom Rating Scale (GSRS)], the stool frequency and consistency according to the Bristol Stool Chart.

Patients were followed throughout the study for safety by physical examination, clinical laboratory tests, adverse events, and compliance with study treatment administration and symptoms. From the beginning until the end of the study the adherence to both the GFD and the study protocol were checked weekly by telephone interview and any violation of the protocol (included drugs) was registered. At the end of the 2^{nd} and 4^{th} treatment week, patients were monitored for anti-tTG2 and EMA antibodies, and those with positive results underwent a repeat endoscopy for checking the integrity of the duodenal mucosa: in the event of any deterioration of histology, patients were considered as treatment failures. Patients with negative serology were kept on treatment for the scheduled 12 weeks. At 12^{th} week, patients had to return for a clinical examination, a repeat blood drawing for anti-tTG2, anti-EMA, anti-gliadin (AGA) both IgG and IgA antibodies checking, routine chemistry, complete hematology work up including serum iron, phosphorus, folate, and vitamin D3 measurements. In addition, a new, repeat endoscopy was scheduled for all enrolled patients with the intent to acquire information on the histologic status of the duodenal mucosa.

Ethical approval was granted by the local Ethics Committee (46/CE). The study was registered on Clinicaltrials.gov (Identifier: NCT03137862).

Randomization

Once the normal serology at the initial evaluation was obtained, patients were randomized 1:2:2 to receive treatment with either placebo (bread prepared from corn flour), or the experimental foodstuff (bread containing 3 or 6 g of the Gluten Friendly^{TM}). The overall schema of the study is shown in Fig. 4.1. All patients were instructed to maintain their usual GFD. All study participants, care providers, data managers, and study personnel remained blinded to study treatment assignment throughout the study end.

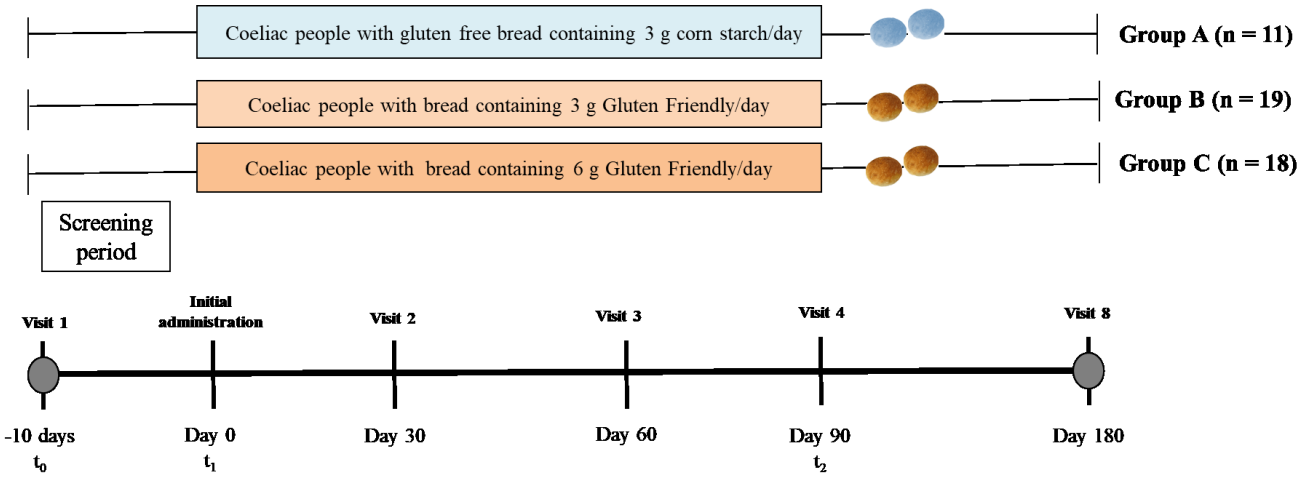


Fig. 4.1. Therapeutic intervention study schema. Patients were randomized to receive bread containing either 3g of "Gluten Friendly™", 6 g of "Gluten Friendly™", or gluten free bread containing 3g of cornstarch as a placebo, daily and for 12 weeks. Upper gastrointestinal endoscopy with duodenal mucosal biopsies was performed on day -10 (baseline) and at post-treatment on day 90.

2.2 Study Treatment

Raw materials and Gluten Friendly™ flour

The wheat kernels (mixtures of soft Italian grains containing 6% of gluten) used in this study to prepare "Gluten Friendly™" (GF) flour were supplied by the Casillo Group S.p.A. (Corato, Italy). Grains, previously harvested and threshed, were treated with microwave energy according to the patented "Gluten Friendly ™" technology (Lamacchia et al., 2013; 2015). The technology has since been further improved (Lamacchia, 2016). Specifically, 100 g of cleaned wheat grains were dampened to 15–18% moisture; moisture was evaluated using an Halogen Moisture Analyzer (Mettler Toledo HB43-S, Switzerland). The seeds were heated with microwaves (DeLonghi, Italy, for about 1 min between 1000 and 750 W), followed by a phase of slow evaporation of the water content. Rapid heating and slow evaporation were repeated up to a final temperature of 80–90°C, as measured with a thermal camera (FLUKE i 20, Italy), and a moisture level of 13–13.5%. After microwave treatment, the Gluten Friendly™ wheat kernels were cooled and dried at room temperature (24°C) for 12–24 h and then ground using an automatic laboratory mill MCKA (Bühler AG, Azwil, Switzerland, diameter of grid 118–180µm) to produce the "Gluten Friendly™" flour.

Gluten Friendly™ Bread preparation

The "Gluten Friendly™" bread administered to patients was produced in the laboratories of Casillo Group S.p.a. (Corato, Ba) in accordance with best practices and using the following recipe: a) bread containing 6 g of "Gluten Friendly™": 100 g of "Gluten Friendly™" flour (containing 6 g of Gluten Friendly™), 25% yeast, 18% salt, 65% water; b) bread containing 3 g of Gluten Friendly™: 50 g of "Gluten Friendly™" flour (containing 3 g of "Gluten Friendly™"), 50 g of corn/rice starch, 25%

yeast, 18% salt, 65% water; c) gluten free bread: 100 g of corn/rice starch, 25% yeast, 18% salt, 65% water. Doughs were prepared by mixing all ingredients in a dough mixer, then leavened for approximately 20 min (at 28/30°C and 70% humidity) and finally shaped into little buns of 50 g each. The leavened bread was placed on Teflon perforated trays and baked in an oven at 220°C for 20 min. The bread was made fresh daily, packed in a controlled atmosphere (N_2/CO_2, 30/70%) and delivered (2 buns of 50 g) home to all patients involved in the study.

2.3 Methods

Endoscopic technique

UGE was undertaken by 1 or 4 experienced endoscopists, all of whom were staff at the Endoscopic Unit of the Division of Gastroenterology, Fondazione IRCCS "Casa Sollievo della Sofferenza" Hospital, in San Giovanni Rotondo, Italy. An independent observer checked all procedures performed by the endoscopists, ensuing consistency in videorecording the macroscopic appearance of the descending duodenum and biopsy sampling. UGE was performed with an Olympus GIF240 (Olympus, Tokyo, Japan), with duodenal biopsies performed distally to the Vater's papilla by using Olympus FB-240K biopsy forceps (2.8-mm standard oval with a needle). All procedures were performed by the use of pharyngeal local anesthesia and conscious sedation, with deep sedation under strictly controlled anesthesia in selected cases. The presence of macroscopic features suggestive of CD (mosaic pattern, scalloping, loss of folds, nodularity and visible submucosal vascular pattern) were recorded in all cases.

Biopsies and histology

In total, 7 biopsy specimens from the distal second part of the duodenum using a one-bite per-pass technique were taken as per the trial protocol. Four specimens were immediately immersed in formalin and shipped to the Jilab Inc. Tampere, Finland where centrally processed to ensure uniform specimen and orientation.

Two of the seven biopsy specimens were used for tissue EMA evaluation (Antiendomisium biopsy, Eurospital, Trieste, Italy).

Histology: Routinely formalin-fixed samples were processed for paraffin blocks using a standard paraffin-infiltration protocol. Each biopsy was embedded in a separate paraffin block under dissection microscope, aiming at a perpendicular cutting plane to the mucosal lumen surface according to Taavela et al. (2013). Six tissue sections (thickness 3-4 μm) were cut on SuperFrost Plus microscope objective slides from each block by tilting the block 5-10 degrees between each cut. For Vh/CrD measurements the slides were stained with hematoxylin and eosin. A crucial step in the procedure is

to train the laboratory technicians to obtain correctly oriented cuttings of biopsy specimens for morphometric evaluation.

Immunohistochemistry: Immunohistochemistry of IELs was done with anti-CD3 antibody (Clone SP7, REF: RM-9107-S1, Thermo Fisher Scientific, Waltham, MA. Diluted 1:300). Crypt cell proliferation index was determined with anti-Cyclin B1 Ab-4 antibody (Clone GNS11, REF: MS-869-P1, NeoMarkers Inc., Fremont, CA. Diluted 1:100). Expression of MUC2 in the villus epithelium goblet cells was visualized with anti-MUC2 antibody (Clone BSB-45, REF: BSB 6160, Bio SB Inc., Santa Barbara, CA. Diluted 1:250). A standard IHC protocol using high-pH antigen retrieval (Tris-EDTA buffer, pH 9) and a peroxidase-polymer based detection kit (Histofine High Stain HRP (MULTI), REF: 414483F, Nichirei Biosciences Inc.) was employed. Diaminobenzidine (DAB) was used as chromogen and hematoxylin as counterstain. Stainings were carried out using an automated stainer platform (LabVision Autostainer). Slides were scanned as high-resolution whole-slide images at a resolution of 0.16 µm per pixel (Jilab Inc., Tampere, Finland). Areas containing the strongest labeling at low magnification were chosen for digital image analysis (hotspot sampling). Three to six villi covering at least 300 enterocytes were counted for IELs. Counting was done automatically using the Celiac Slide Analyzer (Jilab Inc, Tampere, Finland), which is a modification of a multi-purpose IHC cell counting software (Tuominen and Isola., 2010). The results were reported as the number of IELs and MUC2+ cells per 100 enterocytes or as percentage of proliferating Cyclin B1+ crypt cells. Measurements were independent of clinical information.

Digital histomorphometry: All slides were scanned as whole slide images using SlideStrider scanner at resolution 0.28 µm per pixel (Jilab Inc., Tampere, Finland). Images were stored as JPEG2000 files in the image server and viewed via internet with web-based client software developed for this study (Celiac Slide Viewer). Villus height and crypt depth were measured digitally by drawing polylines. Two academic observers (JT, AP) analyzed all slides independently and an average of the measurements was used as the final result for Vh/CrD ratio. CD3 positive intraepithelial T-lymphocytes were enumerated with the AutoIEL software, and the results were expressed per 100 enterocytes. Proliferating Cyclin B1 staining-positive crypt epithelial cells (out of total) were enumerated with ImmunoRatio 2.5 software (Tuominen et al., 2010). At least 300 cells were counted in both cases.

Serologic data

Blood specimens drawn at baseline, during and at the end of the study period were analyzed using routine assays. For celiac serology, an enzyme–linked immunosorbent assay from INOVA Diagnostics (San Diego, California, United States) was used to measure ANTI-tTGA IgA and IgG

levels, and EMA. Each serologic test was compared with its reference interval and analyzed as a binary variable (i.e. elevated or not).

Cytokines: Plasma samples drawn at baseline, during and at the end of the study period were immediately stored at −80°C at Fondazione IRCCS Casa Sollievo della Sofferenza Hospital. The premixed multiplex beads of the Bio-Plex human cytokine Human 27-Plex Panel (Bio-Rad Laboratories, Milan, Italy), which included twenty-seven cytokines and chemokines [IL-1b; IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 (P70), IL-13, IL-15, IL-17, Basic FGF, Eotaxin, G-CSF, GM-CSF, IFN-ɣ, IP-10, MCP-1 (MCAF), MIP-1a, MIP-1b, PDGF-BB, RANTES, TNF-α, VEGF] was used in presence of 30 µl of plasma. All the plasma samples and standards were run in duplicate and fluorescent signals were read by using Biorad 200 system (Bio-Rad).

Intestinal Permeability

Sample Collection: All Celiac patients participants to the study were provided guidelines for dietary restrictions. In the morning of the test, the patients drank a solution containing 5 g of lactulose, 1 g of mannitol and 20 g of saccharose in 200 mL of deionized water. Urine samples were collected for the next 5 hours in the collecting bottle in presence of 1mL of chlorhexidine (1mg/mL) as antimicrobial agent. Total urine volume was measured, and several 10 mL aliquots were stored at −20° C until the analysis. Urine samples were allowed to thaw at room temperature, then stirred for 1min using a vortex mixer, and then were centrifuged at 5000 g for 4 min to remove the sediment.

To 50 μL of urine samples, controls and standards were added 450 μL of internal standard solution and, after being mixed, a 200 μL aliquot was transferred into a glass vial for the injection to HPLC-MS/MS.

Instrumentation: Measurements were performed on a API 3000 Tandem Mass Spectrometer (AB Sciex, Toronto, Canada) equipped with a turbo ion spray source. Quantification was achieved by using either the multiple reaction monitoring (MRM).

Chromatographic Conditions: The HPLC separation was performed using a 150 × 2 mm, Luna 5 μmNH2 100˚A column (Phenomenex, USA) operating at a flow rate of 300 μL/min, and eluted with a 4min linear gradient from 70 to 30% acetonitrile in water. The oven temperature was set at 40°C. The injection volume was 5 μL, and the total analysis time was 9 min.

Mass Spectrometer Conditions: The ESI source operates in a negative mode. The capillary voltage was set to 3500 V at a temperature of 300°C. The source of the gas was set as follows: nebulizer gas 8 (arbitrary units), curtain gas 7 (arbitrary units) and collision gas 4 (arbitrary units).

Each multiple reaction monitoring (MRM) transition was collected at a resolution of 0.7 amu full width half maximum (FWHM) in the first quadrupole, with a scan time of 0.1 s. The tube lens and

collision settings were established individually for each compound. The conditions for the detection of lactulose, mannitol, and raffinose were obtained by direct infusion of a standard solution (10 μg/mL) in line with the HPLC at initial mobile phase conditions.

Method Validation: To validate the method linearity, LOQ, imprecision, accuracy, recovery, and matrix effect were assessed as previously described in Gervasoni J. et al., 2018. The within-run precision and accuracy ranged from 0.9 to 3.2% and 98.0 to 102.0%, respectively. The between-run precision and accuracy ranged from 2.2 to 4.7% and 96.2 to 101.3%, respectively.

Microbiome analysis

Fecal samples were immediately frozen into a sterile container by the participants in their home freezer at −20°C and subsequently stored at −80°C at Fondazione IRCCS Casa Sollievo della Sofferenza Hospital. Genomic DNA was extracted using DNA powerfecal Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. DNA quantity was examined for each sample using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc. Somerset, USA). Microbial diversity analysis in the fecal samples was studied by sequencing the amplified V3 to V4 hypervariable region of the 16S rRNA gene on the MiSeq (Illumina, San Diego, CA, USA) platform. PCR primers and conditions are essentially as outlined in the Illumina 16S Metagenomic Sequencing Library preparation guide (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf) (Illumina) with the following exceptions: for the initial 16S PCR, the process was performed using Taq Phusion High-Fidelity (Thermo Fisher Scientific) in 25 µl reaction volumes, and 25 cycles were used in the PCR.

Subsequently, the amplicons were purified using AMPure XP beads (Beckman Coulter, Milan, Italy). After that, was performed the ligation of the dual indexing adapters, in presence of Nextera XT Index Primer1 and 2 (Illumina), Taq Phusion High-Fidelity (Thermo Fisher Scientific), and 5 µL purified DNA according to the manufacturer' instructions. The products were purified using AMPure XP beads to create the final cDNA library.

Libraries concentration and fragment size were measured using a fluorometric based system (Qubit dsDNA BR Assay System; Thermo Fisher Scientific) and the Agilent 2200 TapeStation Bioanalyzer (HS D1000, Agilent Technologies, Santa Clara, CA, USA), respectively. Equal amounts of cDNA libraries were pooled, denatured with NaOH, diluted with hybridization buffer to 7 pM following the Illumina protocol, spiked with 20% PhiX (Illumina). The libraries was loaded into a flow cell V2 (500 cycles) by paired-end sequencing (2 × 250) (Illumina) and sequenced with MiSeq (Illumina) according to the manufacturer's recommendations.

Quantification of Gluten in stools

Fecal samples were immediately frozen into a sterile container by the participants in their home freezer at −20°C and subsequently stored at −80°C at Fondazione IRCCS Casa Sollievo della Sofferenza Hospital. The concentration of GIP (33-mer) in stools was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using the iVYDAL In Vitro Diagnostics iVYLISA GIP-S kit (Biomedal S.L., Seville, Spain) in duplicate, following the manufacturer's guidelines. The optical absorbance was measured at 450 nm using an ELISA reader (Synergy HT, BioTek Instruments, Winooski, VT).

Symptoms assessment

Symptoms of celiac patients were assessed during gluten free diet (before the trial), after 4, 8, 12 weeks of ingestion of bread containing Gluten Friendly (during the trial) and at the end of the trial. Symptoms were registered by telephone interview using the Celiac Symptoms Index (CSI) Questionnaire (Leffler et al., 2009) and the Gastrointestinal Symptom Rating Scale (GSRS) (Revicki et al., 1998). Stool frequency and consistency were registered with the Bristol Stool Chart (Heaton et al., 1992; Srinivas et al., 2019).

2.4 Statistical analysis

The sample size was pragmatic and was calculated to assess the safety and tolerability of the GF products while minimizing unnecessary participant exposure. All statistic tests were performed through the software Statistica for Windows, ver. 12.0 (Statsoft, Tulsa Oklha.).

Symptoms

CSI results were analyzed through the non-parametric test of Friedman ($P<0.05$). The groups of the study (A, B or C) and the time (immediately before the trial, after 4, 8, 12 weeks, and after the trials) were used as categorical predictors.

Preliminary analysis on serology and histology

The values of Vh/CrD, cytokines (2, 4, 6, 8, IFN, TNF), IELs, MUC2, CB1, G12 detection, gut permeability and antibodies were analyzed by means of one-way analysis of variance (ANOVA) and Tukey's test or through the not-parametric test of Friedman if data distribution did not show a normal trend. The critical level of P was set to 0.05.

k-means and second statistic analysis

Vh/CrD, IELs, MUC2, CB1 and 33-mer detection were used as input variables to run a k-means. MUC2 and CB1 were preliminarily standardized as increase after the trial (%), as follows:

$$I(\%)=\frac{E-B}{B}*100$$

Where E is the value at the end of the trial and B at the beginning; 33-mer detection was reported as percentages of negative subjects (i.e. subjects where 33-mer was not detected).

For k-means clustering, the parameter k (number of cluster) was set to 3, while interactions were at least 10. The initial inter-cluster centers were evaluated by sorting the distance and taking observations at constant intervals.

Microbiota analysis

The readings of each family, genus or species were modeled as recovery detection (%) on the total number of reading for each subject; then, the changes on the recovery detection were evaluated as difference between the recovery after and before the trails.

These values were analyzed through the Friedman test ($P<0.05$) to point significant differences ($P=<0.05$).

Combined index of pro-inflammatory microbiota

The index of pro-inflammatory microbiota was evaluated by combining the detection of *Slackia* and *Sutterella*; each subject of clusters of k-means (black, grey, sub-cluster white and sub-cluster gluten free) received a code as follows: 0, if *Slackia* and *Sutterella* were not detected; 50%, if only a genus was detected; 100%, if both genera were detected. The scores were summed and divided for the number of subjects included in each cluster. The statistical differences amongst clusters were pointed out through the chi-square test ($P<0.05$).

3. Results and Discussion

Forty-eight patients were enrolled in the therapeutic intervention study between April and September 2017 and randomized to 1 of 3 groups receiving 100 g of bread containing 0 g (group A), 3.0 g (group B), 6.0 g (group C) of Gluten Friendly™, daily, divided in 2 doses, for 12 weeks (Fig. 4.1). The mean age was 38 years (range, 15-75 years) and 28 patients were women; patient demographics are shown in Table 4.1. All patients in the three different groups completed the study; there were not drop out of the study before completing all the study visits. The efficacy analyses (per protocol) were based

on 19 patients in group B (Gluten Friendly 3 g per day), 18 in group C (Gluten Friendly 6 g per day) and 11 patients in the placebo group A.

		Gluten Friendly	
	placebo	3.0 g	6.0 g
N of patients	11	19	18
Female	7	11	10
Age in years, mean (ds)	38.6 ± 21.1	35.0 ± 15.3	39.1 ± 15.1
<20	3	3	3
20-49	5	11	10
≥ 50	3	5	5
Measurement			
Height, means (SD) in cm	168.5 ± 9.4	168.9 ± 9.6	168.4 ± 10.1
BMI mean (SD)	21.7 ± 2.4	22.8 ± 3.8	23.5 ± 3.1
<20	2	4	2
20-24.9	9	11	9
25-29.9		3	6
≥ 30		1	1
Duration of GFD in years			
mean (SD)	7.0 ± 6.7	12.3 ± 6.6	11.4 ± 9.2
1-1.9	1		1
2-3.9	2	1	2
4-5.9	2	4	4
6-7.9	2	2	1
>8	4	12	10
HLA			
DQ2	9 (82%)	16 (84%)	17 (94%)
DQ8	1 (9%)	0 (0%)	1 (6%)
DQ2-DQ8	1 (9%)	3 (16%)	0 (0%)

Table 4.1: Characteristics of randomized patients with celiac disease.

Gluten Friendly™ induces symptoms relief

Gastrointestinal symptoms are very frequent and severe in untreated celiac disease patients, and in general, the treatment with gluten free diet of CD results in significant improvement in the symptoms in symptomatic patients; even though symptoms do not disappear completely (Mustalahti et al., 2002; Paavola et al., 2012; Burger et al., 2017;). Reintroduction of gluten by gluten challenge increases considerably the gastrointestinal symptoms of celiac patients (Lähdeaho et al., 2011; Leffler et al., 2013).

The effects of "Gluten Friendly™" administration on symptoms of celiac patients were analyzed by means of the Celiac Symptoms Index Questionnaire (CSI) and Gastrointestinal Symptom Rating Scale (GSRS), immediately before, during (at weeks 4, 8 and 12), and at the end of the trial. Focusing

on CSI scores (Fig. 4.2), the groups A, B and C were not significantly different at the beginning of the trial, thus confirming the homogeneity amongst the subjects included in the three groups; however, they experienced a significant decrease ($P<0.05$, Friedmann test) after 4 weeks and remained at their lowest value during the trial without significant differences due to time (assessment at week 4, 8 or 12) or group (A, B or C). After the trial, CSI scores increased, although at levels lower than the pre-trial ones.

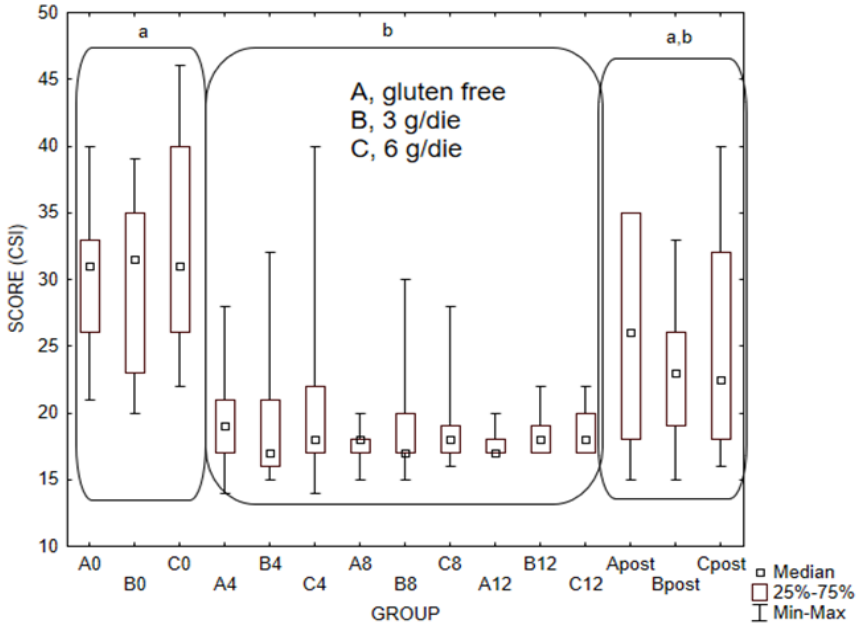


Fig. 4.2: Box-whisker plot on CSI scores on the three groups of the trial (A, 0 g of Gluten per day; B, 3 g of Gluten Friendly per day; C, 6 g of Gluten Friendly per day). The numbers after the letter A, B, and C indicate the time; 0, before the trial; 4, 8, and 12, at week 4, 8 and 12; post, after the trials. The small letters indicate significant differences (Friedmann test, $P < 0.05$).

These results are in accordance with GSRS (data not shown) and with the stool frequency and consistency registered with the Bristol Stool Chart (data not shown) showing that Gluten Friendly™ is very well tolerated by celiac patients and improves their quality of life inducing symptoms relief. In Leffler's study (Leffler et al., 2013) twenty adults with biopsy-proven coeliac disease participated to a gluten challenge for two weeks at a randomly assigned dose of 3 or 7.5 g of gluten/day. Gastrointestinal symptoms assessed by Celiac Symptom Index (CSI) (Leffler et al., 2009) and Gastrointestinal Symptom Rating Scale (GSRS) (Revicki et al., 1998) increased significantly by day 3 and returned to baseline by day 28. No differences were seen between the two gluten doses. Lähdeaho et al. (2011) challenged twenty-five celiac disease adults with 1-3 g or 3-5g doses of gluten daily for 12 weeks. Gastrointestinal symptoms leading to premature withdrawals in seven cases and 71% out of celiac disease patients experienced mild to moderate abdominal symptoms upon gluten challenge. Lähdeaho et al. (2014), in an intervention study, challenged adults with biopsy-proven

celiac disease randomly assigned to groups given ALV003 or placebo together with the daily gluten challenge (2 g) for 60 days. In the efficacy-analysis population there were consistent increases in overall GSRS scores from baseline through day 42, although by day 70 the scores returned to baseline. The change, during the challenge period, in overall GSRS, abdominal pain, and indigestion scores trended higher in the placebo patients; all returned to baseline by day 70.

A not full resolution of symptoms in the three groups (A, B, C) before and after the trial, during gluten free diet, can be related to the ongoing ingestion of gluten, either deliberate or inadvertent, causing persistent inflammation in the small-intestinal mucosa (Catassi et al., 2007). The significant decrease of the placebo group A has been interpreted as a consequence of the strict monitoring of the GFD imposed by the study protocol that, among other things, provided, every week, patients with Biocard Celiac test for rapid screening of the immunoglobulin A (IgA) anti-tTG2.

Gluten Friendly induces a clustering of mucosal histology and inflammation

The gold standard for celiac disease diagnosis is the finding of gluten-induced small intestinal mucosal injury (Walker-Smith et al., 1990; Rostom et al., 2006). The mucosa will heal upon the introduction of a gluten-free diet and the mucosal damage will reappear if gluten is reintroduced (Walker-Smith et al., 1990). Extensive time-course studies have provided evidence that during gluten challenge an inflammatory process with a dose-dependent accumulation of intraepithelial lymphocytes (IELs) is followed by mucosal villous atrophy with crypt hyperplasia (Marsh et al., 1995).

The effect of Gluten Friendly™ on mucosal histology and inflammation of celiac patients was studied by analyzing small-bowel morphology (Vh/CrD), densities of CD3+ intraepithelial lymphocytes (IELs) and other important markers of CD inflammation such as MUC2 and CB1, gluten antibodies, cytokines, and intestinal permeability.

G12-ELISA immunoassay was used to assess the absence/presence of gluten (33-mer peptide) in stools of placebo group (A) and groups fed with Gluten Friendly (B, C), respectively and to monitor the adherence to gluten free (group A) or "Gluten Friendly™" diet (group B and C).

Fig. 4.3 shows the results of Vh/CrD. Statistic pointed out a significant decrease of Vh/CrD from the group A (placebo group) to the groups B and C; however, boxes and plots suggested a strong variability within each group, mainly in the groups B and C.

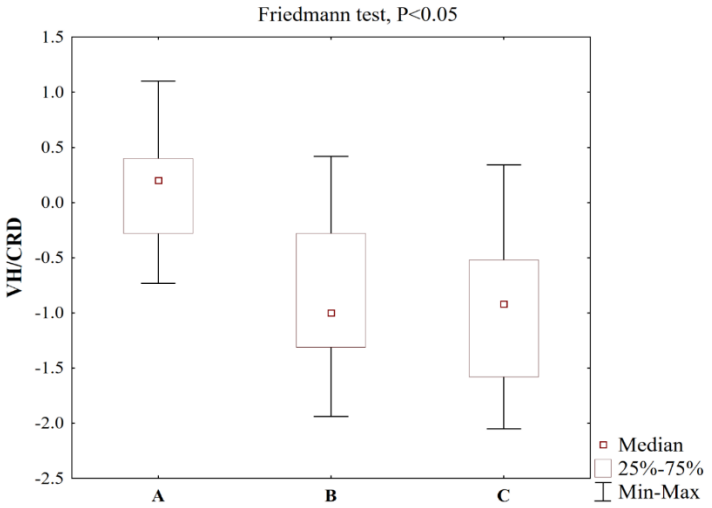


Fig. 4.3: Vh/CrD at the end of the trial in groups A, B, and C.
A, 0 g of Gluten per day; B, 3 g of Gluten Friendly per day; C, 6 g of Gluten Friendly per day.
Morphometric analysis measuring Vh/CrD was made in well-oriented biopsy samples. During the gluten-challenge, a decrease in Vh/CrD of 0.5 or more was considered significant and equivalent to clinical gluten-sensitivity. Measurements were independent of clinical information.

IELs are in Fig. 4.4; the median values were 26%, 38% and 57% for the groups A, B, and C respectively; however, the differences were not significant and the box-plots suggested a strong variability within the trial groups.

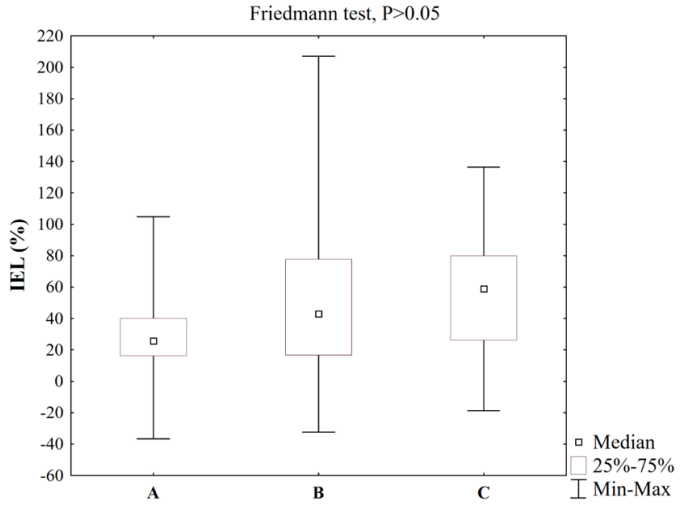


Fig 4.4: IELs increase at the end of the trial in groups A, B, and C.
A, 0 g of Gluten per day; B, 3 g of Gluten Friendly per day; C, 6 g of Gluten Friendly per day.
Immunohistochemistry of IELs was done with the anti-CD3 antibody. Three to six villi covering at least 300 enterocytes were counted for IELs. Counting was done automatically using the Celiac Slide Analyzer (Jilab Inc, Tampere, Finland). CD3 positive intraepithelial T-lymphocytes were enumerated with the AutoIEL software, and the results were expressed per 100 enterocytes. After the gluten challenge, an over 30% increase in IEL counts was considered significant and equivalent to clinical gluten-sensitivity. Measurements were independent of clinical information.

MUC2 and CB1 were analyzed by one-way ANOVA (Figs. 4.5 and 4.6); the differences amongst the groups were not significant and, as reported above, each group experienced a high variability. The

same records were found for cytokines (as an examples Figs. 4.7 - 4.10, show cytokines 2, 4, 6 and 8), TNF and IFN (Figs. 4.11 and 4.12) and gut permeability (Fig. 4.13).

EMA and tTGA (antibodies) were never detected in the subjects of group A; while the recovery was 15% and 47% after 1 month, respectively in groups B and C. Then, it increased to 42-68% after 3 months (data not shown).

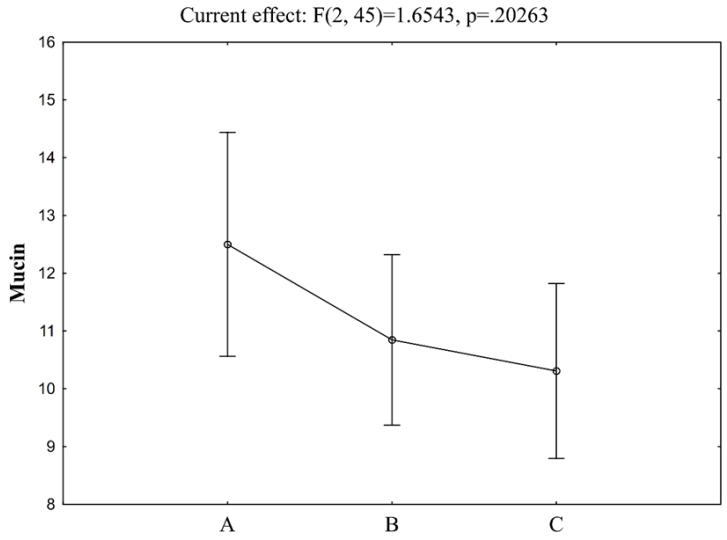


Fig. 4.5: Mucin 2 at the end of the trial in groups A, B, and C.
Bars denote 95% confidence intervals. A, 0 g of Gluten per day; B, 3 g of Gluten Friendly per day; C, 6 g of Gluten Friendly per day. Expression of MUC2 in the villus epithelium goblet cells was visualized with anti-MUC2 antibody (Clone BSB-45, REF: BSB 6160, Bio SB Inc., Santa Barbara, CA. Diluted 1:250). The results were reported as the number of MUC2+ cells per 100 enterocytes. Measurements were independent of clinical information.

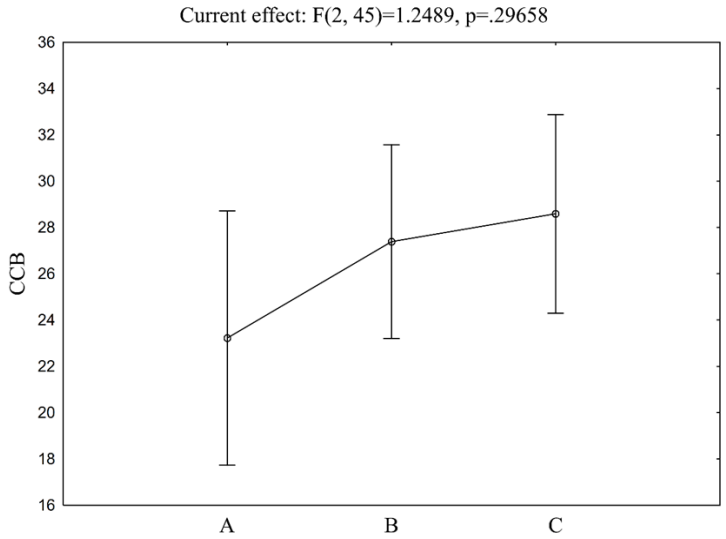


Fig. 4.6: Crypt cell proliferation (CB1) at the end of the trial in groups A, B, and C.
Bars denote 95% confidence intervals. A, 0 g of Gluten per day; B, 3 g of Gluten Friendly per day; C, 6 g of Gluten Friendly per day. Crypt cell proliferation index was determined with anti-Cyclin B1 Ab-4 antibody (Clone GNS11, REF: MS-869-P1, NeoMarkers Inc., Fremont, CA. Diluted 1:100). The results were reported as the percentage of proliferating Cyclin B1+ crypt cells. Measurements were independent of clinical information.

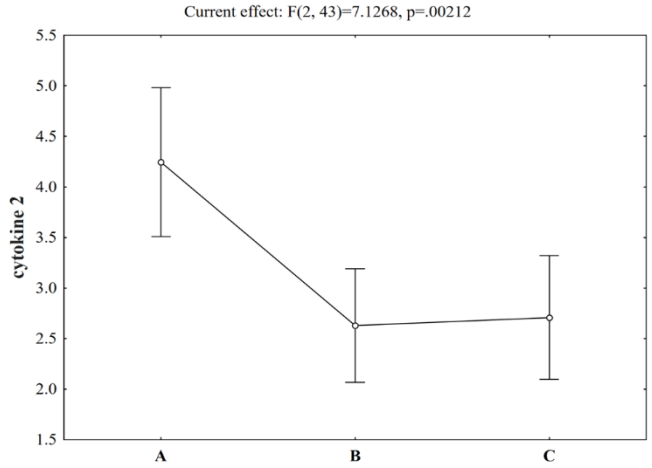


Fig. 4.7: Cytokine 2 at the end of the trial. Bars denote 95% confidence intervals. A, 0 g of Gluten per day; B, 3 g of Gluten Friendly per day; C, 6 g of Gluten Friendly per day.

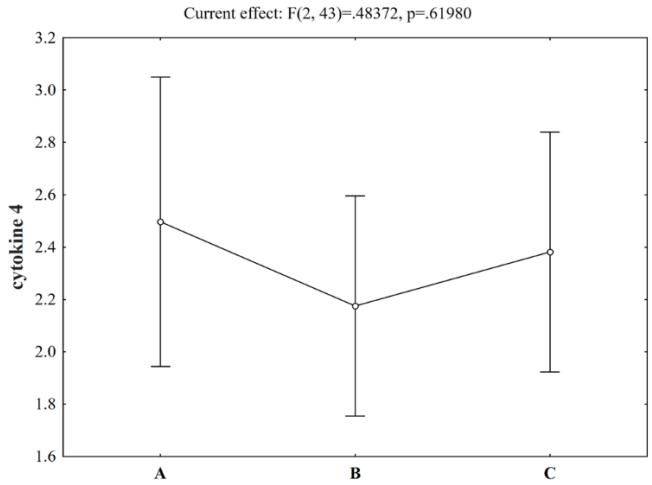


Fig. 4.8: Cytokine 4 at the end of the trial. Bars denote 95% confidence intervals. A, 0 g of Gluten per day; B, 3 g of Gluten Friendly per day; C, 6 g of Gluten Friendly per day.

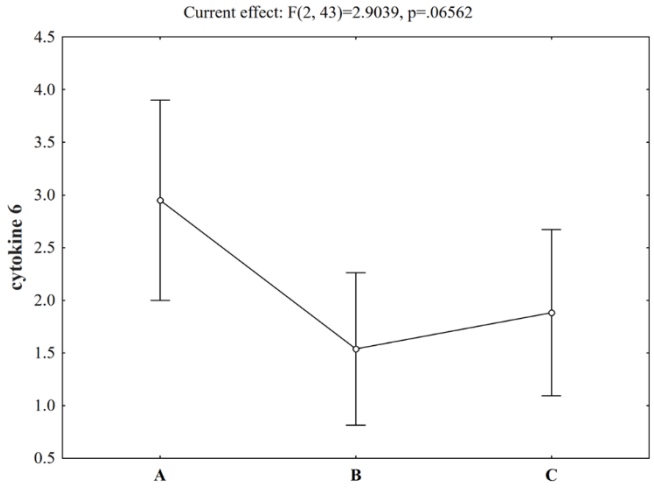


Fig. 4.9: Cytokine 6 at the end of the trial. Bars denote 95% confidence intervals. A, 0 g of Gluten per day; B, 3 g of Gluten Friendly per day; C, 6 g of Gluten Friendly per day.

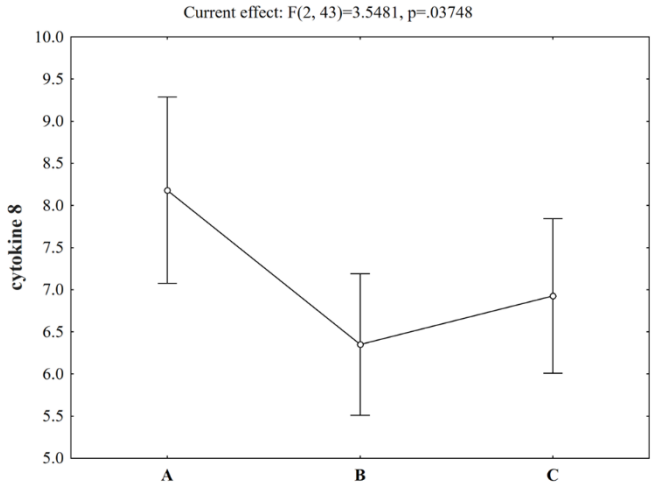


Fig. 4.10: Cytokine 8 at the end of the trial. Bars denote 95% confidence intervals. A, 0 g of Gluten per day; B, 3 g of Gluten Friendly per day; C, 6 g of Gluten Friendly per day.

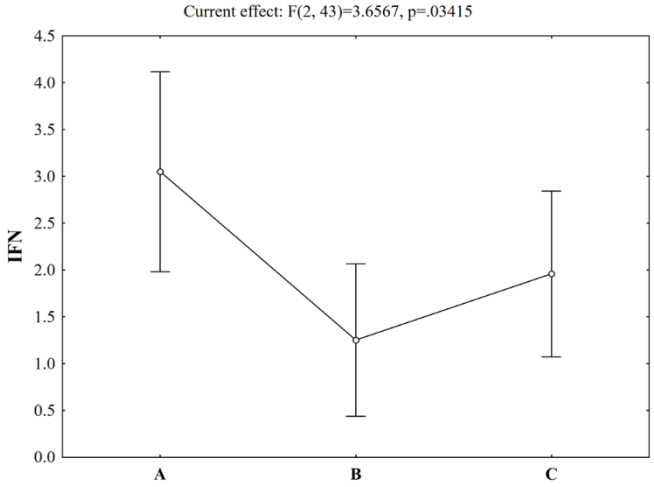


Fig. 4.11: IFN at the end of the trial. Bars denote 95% confidence intervals. A, 0 g of Gluten per day; B, 3 g of Gluten Friendly per day; C, 6 g of Gluten Friendly per day.

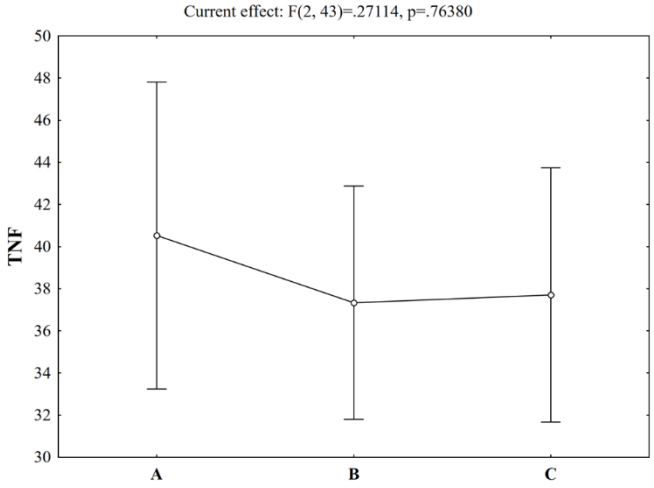


Fig. 4.12: TNF at the end of the trial. Bars denote 95% confidence intervals. A, 0 g of Gluten per day; B, 3 g of Gluten Friendly per day; C, 6 g of Gluten Friendly per day.

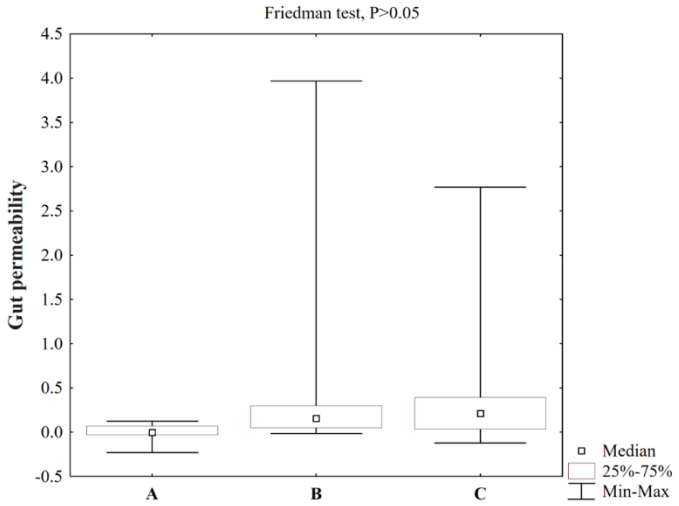


Fig. 4.13: Gut permeability at the end of the trial. A, 0 g of Gluten per day; B, 3 g of Gluten Friendly per day; C, 6 g of Gluten Friendly per day.

As a last assay, G12 detection in stool was studied; as expected, it was never detected in the group fed with gluten free (group A). Considered that gluten is a protein difficult to digest and that "Gluten Friendly™" is new type of gluten with a "new shape" (Lamacchia, 2016) where the primary structure does not change (Lamacchia et al, 2016), the detection in all B and C samples was expected, but it was surprisingly not found in 8 patients from group B (42%) and 10 patients from group C (55%). Moreover, the expected outcome was a strong correlation of both: antibodies/33-mer detection (90-100%) and mucosal injury (reduction of Vh/CrD) /33-mer detection, respectively. However, this kind of correlation was not found, as the recovery of 33-mer was lower than the expected one and a partial uncoupling between antibodies and 33-mer and Vh/CrD and 33-mer was found.

Besides, Vh/CrD was not related and coupled with IELs increase and CB1 production, instead, it was related to MUC2. The proximal small intestinal mucosa of untreated coeliac disease is characterized histologically by loss of the normal villous architecture and crypt hyperplasia. Crypt hyperplasia is associated with increased turnover of epithelial cells, accelerating migration of cells from the crypt base to the villus tip and one of the important marker for this increased turnover is represented by the CB1 (Niranjan et al., 2016). In addition to infiltration by T cells in the lamina propria, there is also an increase in the number of intraepithelial lymphocytes (IELs) and a reduction in the total number of goblet cells producing MUC2 in the duodenal mucosa of untreated coeliac patients. As a consequence, we were expected a strong correlation between the Vh/CrD decrease and IEL and CB1 increase over a MUC2 decrease. As well as we expected also that the mucosal injury was correlated with an increase of both: celiac subjects gut permeability and cytokines. Several studies have previously reported that gluten affects the gut barrier permeability by releasing zonulin (Fasano et al., 2000) and TNF-α and IL-6, in addition to other proinflammatory cytokines activate cytotoxic CD8+ T cells responsible for tissue remodeling and damage (Agarwal et al., 2016). Furthermore, in the

mucosa of patients with active CD, mRNA coding for IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-18, IL-21, IL-22 are overexpressed (Hansson et al., 1999; Khatkar, 2014; Girard-Madoux et al., 2016; Iacomino et al., 2016; Vorobjova et al., 2019) and proinflammatory cytokines, such as TNF-α and others released by DCs, can disrupt proteic components of TJs via phosphorylation and in turn increase the permeability of intestine (Kelsall et al., 2004; Rescigno, 2008; Moheb-Alian et al., 2016; Felli et al., 2017; Buckley et al., 2018).

On the contrary, the partial correlation of the Vh/CrD with tTG2 and EMA was predictable since it was found that serum tTG2 and EMA often underestimate the degree of Vh/CrD (Silvester et al., 2017). In one of the largest studies to date (Hopper et al., 2008) IgA anti-tTG2 failed to detect 44% of persistent VA (Marsh III) in patients with CD on a GFD for >1 year.

The main conclusion of this step was that groups A, B and C were not significantly different for most parameters, as the subjects of each group experienced a high variability of the data and different trends. Moreover, data suggested an uncoupling between Vh/CrD and IEL, CB1, cytokines, gut permeability and presence of gluten in stools.

As a result, a new hypothesis was done, that subjects tended to cluster depending on "unknown variables" different from the groups of the trial (feeding with gluten free or "Gluten Friendly" bread). To understand how the samples could be grouped, all subjects were included in a unique group and analyzed through K-means by using an approach that could be labeled as "a priori clustering": samples were first grouped and then studied to point out the factors playing a major role.

K-means is unsupervised learning, used to find groups in unlabelled data, with the number of groups represented by the variable K. This statistic assigns each data point to one of K group based on the similarity for some input variables. In order to avoid statistical artifacts, K was set to 3, because the subjects were divided into 3 groups in the trial. Vh/CrD, IEL, mucin production, CB1 production and 33-mer detection were used as input variables. However, IEL, mucin, CB1 production and 33-mer detection were preliminary standardized and converted in relative increase/decrease for CB1, IEL, and mucin or relative detection for 33-mer. The choice of using relative increases depends on the fact that at the baseline the groups of the trial were similar and the hypothesis is that something happened during the trial. Moreover, from a methodological point of view, this step was necessary to carry out a statistic with homogeneous factors.

Permeability and cytokines were not used as categorical factors, because the groups of the trial (A, B and C) did not show significant differences for them. Therefore their use could lead to an increase in the complexity of the dataset with the risk of covering possible differences.

K-means clustered the subjects of groups A, B, and C in a different way; details are in Tables 4.2 and 4.3.

	Cluster 1 (black) (K1)	Cluster 2 (grey) (K2)	Cluster 3 (white) (K3)
VH	-1.07	-1.28	-0.10
IEL	97.80	46.22	13.52
G12	47	36	9
CB1	16.88	20.01	-3.12
MUC2	-13.15	-23.80	15.23

Tab. 4.2: Mean values of the categorical predictors of k-means.
VH, Vh/CrD; IEL, IEL increase (%); G12, detection of G12 (%); CB1, increase of CB1 (%); MUC2, increase of MUC2 (%).

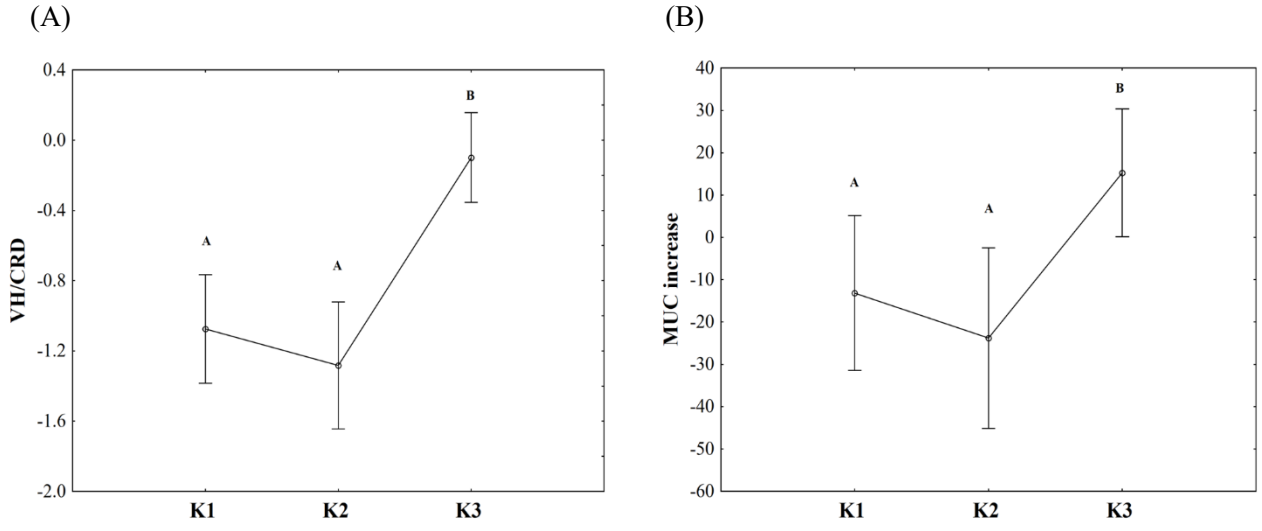
Cluster 1 (or black cluster, K1) included 15 individuals (7 from group C, 6 from group B and 2 from group A), Cluster 2 (or grey cluster, K2) was composed of 11 individuals (5 from C and 6 from B) and Cluster 3 (or white cluster, K3) of 22 individuals (6 from C, 7 from B and 9 from A). Table 4.2 shows the mean values for the different parameters, while Figure 4.14 reports the statistic on them. Vh/CrD (Fig. 4.14A) experienced a 2-class trend, as black and grey clusters (Vh/CrD at -1.07 and -1.28%, respectively) were not different, but they were significantly different to white cluster (Vh/CrD at -0.10%). The same trend was found for MUC2 increase (Fig. 4.14B), with black and grey clusters experiencing a decrease of MUC2 (-13.15 and -23.80%) and white cluster an increase (+15.23%); and CB1 increase (+16.88% and +20.01% for black and grey clusters and -3.12% for white cluster) (Fig. 4.14C).

These results enhanced the idea of a coupling/correlation Vh/CrD, mucin and CB1 production. However, these parameters were not related to IELs, which experienced a different trend. IELs, in fact, showed a 3-class trend, with each cluster different from the others. The mean of IELs was maximum (+97.80%) for black cluster and the minimum for the white one (+13.52%) and an intermediate value for the grey cluster (+46.22%) (Fig. 4.14D).

The 33-mer detection (presence of gluten) showed the trends by homogeneous groups with a maximum, a minimum and a transition zone, that is 91% of subjects of white cluster were negative to G12 (20 subjects: 11 receiving gluten free and 9 Gluten Friendly bread) and 53% for black cluster (8 subjects), while grey cluster was a transition cluster (64% of cluster negative to G12-7 subjects), for a total of 25 subjects receiving Gluten Friendly with non 33-mer in the stool (Fig. 4.14E).

Cluster 1 (black) (K1) 15 individuals: 7 from C 6 from B 2 from A	**Cluster 2 (grey) (K2)** 11 individuals: 5 from C 6 from B	**Cluster 3 (white) (K3)** 22 individuals: 6 from C 7 from B 9 from A
1C	4C	5C
2C	7C	6C
3C	8C	10C
9C	12C	11C
13C	18C	15C
14C	3B	16C
17C	5B	1B
2B	7B	6B
4B	17B	9B
8B	18B	13B
10B	19B	14B
11B		15B
12B		16B
2A		1A
3A		4A
		5A
		6A
		7A
		8A
		9A
		10A
		11A

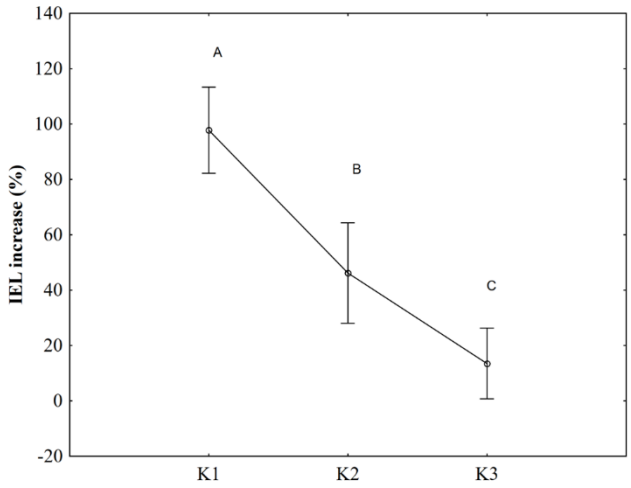
Tab. 4.3: Subjects included in the clusters of k-means.



(C)

Clusters	CB1	Statistical groups	
		I	II
K3-white	-3.12	****	
K1-black	16.88	****	****
K2-grey	20.01		****

(D)



(E)

Clusters	Subjects negative to G12	Statistical groups	
		I	II
K3-white	91%	****	
K1-black	53%	****	****
K2-grey	64%		****

Fig. 4.14: Vh/CrD (A), MUC2 increase (B), CB1 (C) and IELs increase (D), 33-mer absence in stools (E) in the clusters of k-means: mean values. In figures A, B and D the bars represent 95% confidence intervals and the letters in the figures denote significant differences (one-way ANOVA or Friedmann test). In figures C and E, the asterisks denote clusters belonging to homogeneous groups (one-way ANOVA).

Considering only coeliac patients (group B+group C), "Gluten Friendly™" administration induced a clustering of celiac subjects in 3-zones, as follows: 35% black zone (K1), 30% grey zone (K2), 35% white zone (trend as the placebo group) (K3). The statistic pinpointed an uncoupling among some parameters. Namely, Vh/CrD significantly decreased in black and grey zone, but surprisingly it was not related to IEL increase in the grey zone and to the detection of 33-mer in K1 and 2 (black and grey zones). Thus suggesting a positive effect in 35% of patients (white zone) and a possible dynamic situation, with a positive modulation of the inflammation, in 30% of patients (grey zone). The positive effect observed in the white zone is significantly correlated with the 33-mer absence in patient stools. Nevertheless, if this was the only important element involved in the clustering induced by the Gluten Friendly, then it would not be explained i) why, despite the absence of 33-mer in 53% and 64% of

patients, in the black and grey zones, respectively, 90% of them experienced an intestinal damage; ii) why, in the grey zone, the intestinal damage (Vh/CrD), at the end of the three months trial, was uncoupled with inflammation (IEL); iii) why, celiac patients of the grey and black zones, underwent symptoms relief.

Recent studies has hypothesized that CD might be triggered by additive effects of immunotoxic gluten peptides and intestinal dysbiosis (microbial imbalance) in the people with or without genetic susceptibilities (Chander et al., 2018).

Therefore, convinced that the analyzes carried out so far were necessary according to Tampere raccomandations (Ludvigsson et al., 2018) but not sufficient to explain the effect obtained on patients from the administration of Gluten Friendly, we decided to explore and deepen our study on what is now called the "superorganism" (Rinninella et al., 2019): the intestinal microbiota.

Gluten Friendly is digested by celiac on the basis of their microbiota traits

Several researchers stressed the importance of microbiota in celiac disease (Chander et al., 2018) and reported that celiac subjects generally show a typical microbial imprinting with some genera/family prevailing (Gram negative, pro-inflammatory microbiota). As a preliminary result, the microbiota of stool at baseline was analyzed to check if the subjects of the trials were homogeneous or if a difference in the initial microbiota could be responsible for the different outcome to Gluten Friendly™ bread. As a preliminary step, OTU from bioinformatics were converted in numeric code as follows: 0 genus/ family lacking and 1 when the genus/family was recovered.

The initial dataset was a binary dataset showing the recovery detection (%) of each family. The data were analyzed by using as categorical factors both the groups of the trial (A, B and C) and the clusters of k-means (black, grey and white). When k-means clustering was used, the cluster white was divided into two sub-clusters: white (subjects fed by GF bread) and free (subjects fed by gluten free bread).

The differences were not significant, except for two genera, *Slackia* and *Sutterella*, when analyzed by using the clusters of k-means. Fig. 4.15A shows the recovery detection in the clusters: *Sutterella* was found in 66.67% and 40.0% of subjects of black and grey clusters, while the recovery detection was significantly lower in white cluster (Chi-square test, $P<0.05$). *Slackia* was never found in the subjects of white cluster, while the recovery detection was 16.67% in black cluster and 60% in grey cluster, with a significant difference between grey and white clusters ($P<0.05$, Chi-square test).

These recovery values suggested that the initial content of *Slackia* and *Sutterella* could play a role in the different response to GF bread. The interaction between a balanced, stable microbiota and the mucosal immune system could maintain intestinal homeostasis, however, it can be easily disturbed by alterations in the microbial community leading to pro-inflammatory immune responses. This

imbalance is known as dysbiosis and is characterized by a decrease in the abundance of Firmicutes and an increase of Proteobacteria (Mukhopadhya et al., 2012; Shin et al., 2015). An increase in Proteobacteria might contribute to a non-specifical mucosal inflammation due to LPS (Round and Mazmanian, 2009). The genus *Sutterella* belongs to Proteobacteria and its role in healthy subjects is not known; however, some evidence suggests an increase and a role in some pathologies, like atopic dermatitis (Reddel et al., 2019), diabetes (Allin et al., 2018), and antibiotic associated diarrhea (Lv et al., 2017).

Slackia was associated with some diseases, for example, abscesses of intestinal origin (Kim et al., 2010), and some authors stressed its importance in the dysbiosis connected with some pathological situations, like cancer (Coker et al., 2018).

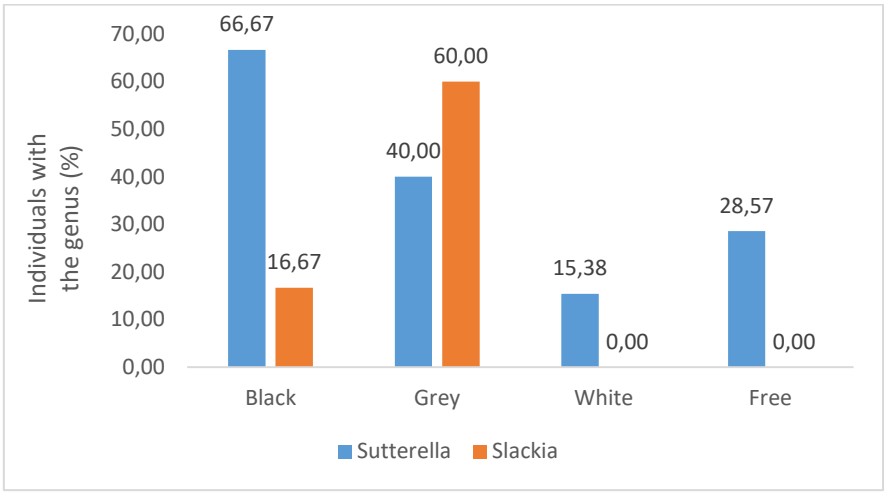
Furthermore, environmental infectious agents compete with the beneficial microbes to adhere to the intestinal mucosa, and after adherence, they disturb intestinal barrier function (tight junctions) by activating different inflammatory pathways at the intestinal mucosal surface (Serena et al., 2007). Disturbed intestinal barrier leads to exposure of intestinal immune cells to the dietary antigens (i.e., gluten).

Based on this literature evidence, our hypothesis was that *Slackia* and *Sutterella* could jointly contribute to the pro-inflammatory response. Thus, it is not important to study each genus alone but to focus on their synergistic additive effect. Therefore, an arbitrary index was designed and used. A ternary code was assigned to each subject: 0, when both *Slackia* and *Sutterella* were not detected; 0.5, when only a genus was found; 1, when both *Slackia* and *Sutterella* were found. Then, the codes were standardized and the index could be read as follows: 0, *Slackia* and *Sutterella* always absent in all subjects; 100, *Slackia* and *Sutterella* found in all subjects of the groups (Tab. 4.4).

The results of this index, called “standardized score of pro-inflammatory genera” are in Fig. 4.15B. the score was 41.67% and 40.0% in the black and grey clusters, while it was significantly lower in white (11.54%) and free clusters (14.29%) ($P<0.05$, Chi-square test).

Some new studies further highlighted the value of gut microbes in the determination of gluten immunogenicity, namely the fact that pathobionts isolated from CD patients cleaved 33-mer peptide in such a manner that it activated gluten-specific T-cells in CD patients (Caminero et al., 2016). Conversely, an enzyme strategy uses microbial proteases and peptidases for gluten detoxification (Pyle et al., 2005; Shan et al., 2005), and current research is focused on the oral administration of microbial endopeptidases with various degrees of tolerance to the gut environment (Caputo et al., 2010; Rauhavirta et al., 2011).

(A)



(B)

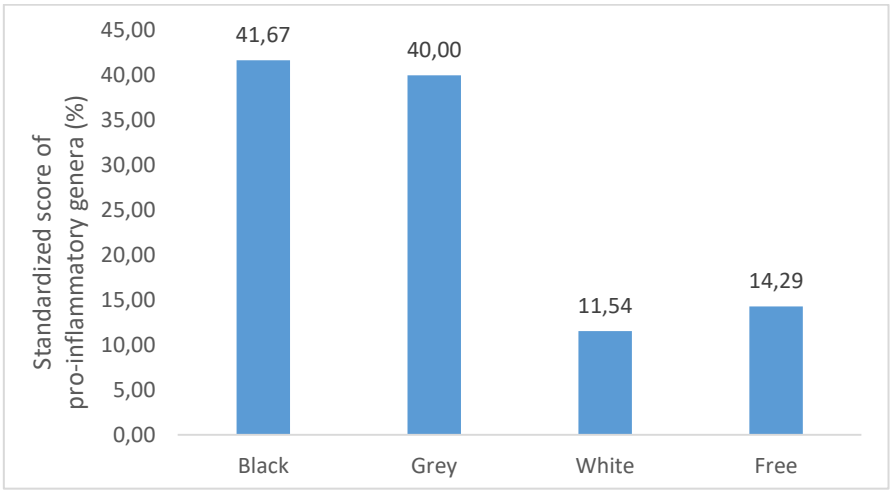


Fig. 4.15: Recovery of *Slackia* and *Sutterella* in the faecal microbiota of the clusters of k-means (A) and pro-inflammatory score (B).

By virtue of this, the presence of *Slackia* and *Sutterella* can also clarify the absence of 33-mer in the white zone (K3) and the partial presence in the other two zones (K1 and 2).

It can be postulated that "Gluten Friendly ™", being gluten with a "new shape", in presence of a healthy microbial ecology could be completely digested by celiac gut microbiota endopeptidases without detrimental effects for the intestine as it happened in K3.

beginning

1 found
0 not found

	Slackia	*Sutterella*	score
K1	1	1	1
K1	0	1	0.5
K1	0	1	0.5
K1	0	1	0.5
K1	0	0	0
K1	0	0	0
K1	0	1	0.5
K1	0	1	0.5
K1	0	1	0.5
K1	1	1	1
K1	0	0	0
K1	0	0	0

5

	Slackia	*Sutterella*	score
K2	1	1	1
K2	0	0	0
K2	1	0	0.5
K2	0	0	0
K2	1	1	1
K2	1	0	0.5
K2	0	1	0.5
K2	0	0	0
K2	0	0	0
K2	0	1	0.5

4

	Slackia	*Sutterella*	score
K3	1	0	0.5
K3	0	1	0.5
K3	0	1	0.5
K3	0	0	0
K3	0	0	0
K3	0	0	0
K3	0	0	0
K3	0	0	0
K3	0	0	0
K3	0	0	0
K3	0	0	0
K3	0	0	0
K3	1	0	0

1.5

	Slackia	*Sutterella*	score
free	0	0	0
free	0	1	0.5
free	0	0	0
free	0	0	0
free	0	0	0
free	0	1	0.5
free	0	0	0

1

	Individuals	Sum of scores	Standaradized score
K1	12	5	41.67
K2	10	4	40.00
K3	13	1.5	11.54
free	7	1	14.29

Tab. 4.4: Evaluation of pro-inflammatory score.

In fact, a healthy microbial ecology (homeostasis), where there is a balance between beneficial bacteria and harmful bacteria, prevents the activation of inflammatory pathways and favorite the immune cells tolerance (Smith et al., 2013; Furusawa et al., 2015; Takiishi et al., 2017; Wang et al., 2019).

At the same time, the presence of *Slackia* and *Sutterella* in K1 and K2 did not allow all celiac subjects (47% and 36%, respectively) to digest completely and correctly "Gluten Friendly™". Furthermore, they predisposed intestine to inflammation (Serena et al., 2017) so that, even traces (under 40 ppm; not detectable by G-12 immunoassay) of 33-mer, maybe present in 53% and 646% of patients in the black and grey zones (K1 and K2) respectively, became detrimental for gut. This would explain the uncoupling parameters in K1 and 2 about histological injury and presence/absence of 33-mer, but could also explain the different sensitivity of celiac patients to a very low amount of gluten (under 20 ppm) (Catassi et al., 2007).

Gluten Friendly shapes celiac microbiota towards intestinal homeostasis

The last step of this research was a focus on the changes of microbiota as a result of GF supplementation, thus the data should be read as recovery variation (increase or decrease) after 3 months. For this kind of results, it is not possible to assume that they follow a normal distribution, therefore they are reported as box-plot picture with medians, and quartiles.

Fig. 4.16A shows the changes in the recovery of *Agathobacter* spp. In the black cluster, the median value of the change was -0.27%, while 3rd and 1st quartiles were 0 and -1.25% (box) and the minimum value was -3.1%. This kind of distribution suggests that subjects of the black cluster (at least 75% of them) experienced a decrease of *Agathobacter* population after 3 months. A similar trend was found for the grey cluster, showing 3rd and 1st quartiles of respectively 0% and -0.8% and a minimum value of -2.1%. On the other hand, statistic revealed significant differences for the white cluster, with a median value of 0.4% and 1st and 3rd quartiles from 0 to 0.8% and a maximum value of 4.3%. The range 1st quartile/maximum (from 0 to 4.3%) suggests that for this cluster the 75% of subjects experienced an increase of *Agathobacter* spp.

Fig. 4.16B and 4.16C show the recovery variation for *Eubacterium coprostanoligenes* and *Tyzzerella* spp. For *E. coprostanoligenes* the differences were not significant, although black cluster experienced a strong variation, while the box of the white cluster (median, 1st and 3rd quartiles around 0%) suggests possible homeostasis for at least 50% of subjects. *Tyzzerella* spp. was in homeostasis for the white and free clusters (box, maximum and median to 0%), while black and grey clusters experienced a significant variation with an increasing trend; the differences between white/free clusters and grey/black clusters were significant (P = 0.0488).

The last two box-plot figures (Fig. 4.16D and 4.16E) show the changes for *Lactobacillus* spp. and *Intestinimonas* spp. *Lactobacillus* spp. experienced an increase (P = 0.0217) in the grey cluster (median and 1st quartile 0.03%; 3rd quartile, 0.15%; maximum, 1.0%) (Fig. 4.16D). Finally, *Intestinimonas* spp. showed an increasing trend in the black cluster (Fig. 4.16E).

Lactobacillus is a well-known genus with various beneficial properties. It can contribute to gut re-equilibration and shape the microbiota towards homeostasis, with a protective effect against some diseases (Chen et al., 2015; Zhang et al., 2019). Moreover, *Lactobacillus* might promote the homeostasis of gut mucus layer through producing L-Orn. L-Orn stimulates Trp metabolism to produce AhR ligands in gut epithelial cells, which induce accumulation of RORγt (+) IL-22(+) ILC3 in gut tissues (Qi et al., 2019).

Their significant increase observed in the grey zone is undoubtedly attributable to the administration of Gluten Friendly, since the GF had already shown, *in vitro*, (Bevilacqua et al., 2016; Costabile et al., 2017) an effect on the growth of Lactobacilli and on their metabolism (bifidogenic effect).

Lactobacilli increase in the grey zone elucidates why the intestinal damage (Vh/CrD) at the end of the three months trial was uncoupled with inflammation (IEL) and solve the question of the relief of symptoms in the celiac patients at least in the grey zone. It can be supposed that, although in this zone the microbiota was not in homeostasis, the GF, correctly digested, was used as a source of energy to Lactobacilli that were turning out the inflammation and may be carrying out gut microbiota towards homeostasis and immunological tolerance. In fact, evidence suggests that gut commensal, including *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Clostridium*, and *Streptococcus*, as well as bacterial metabolites, such as butyric acid and propionic acid may program Treg cells in the intestine towards tolerogenicity (Geuking et al., 2011; Ohue et al., 2011; Rodriguez et al., 2012; Furusawa et al., 2015), and help the host with maturation and homeostasis of immune system (Lin and Zhang, 2017).

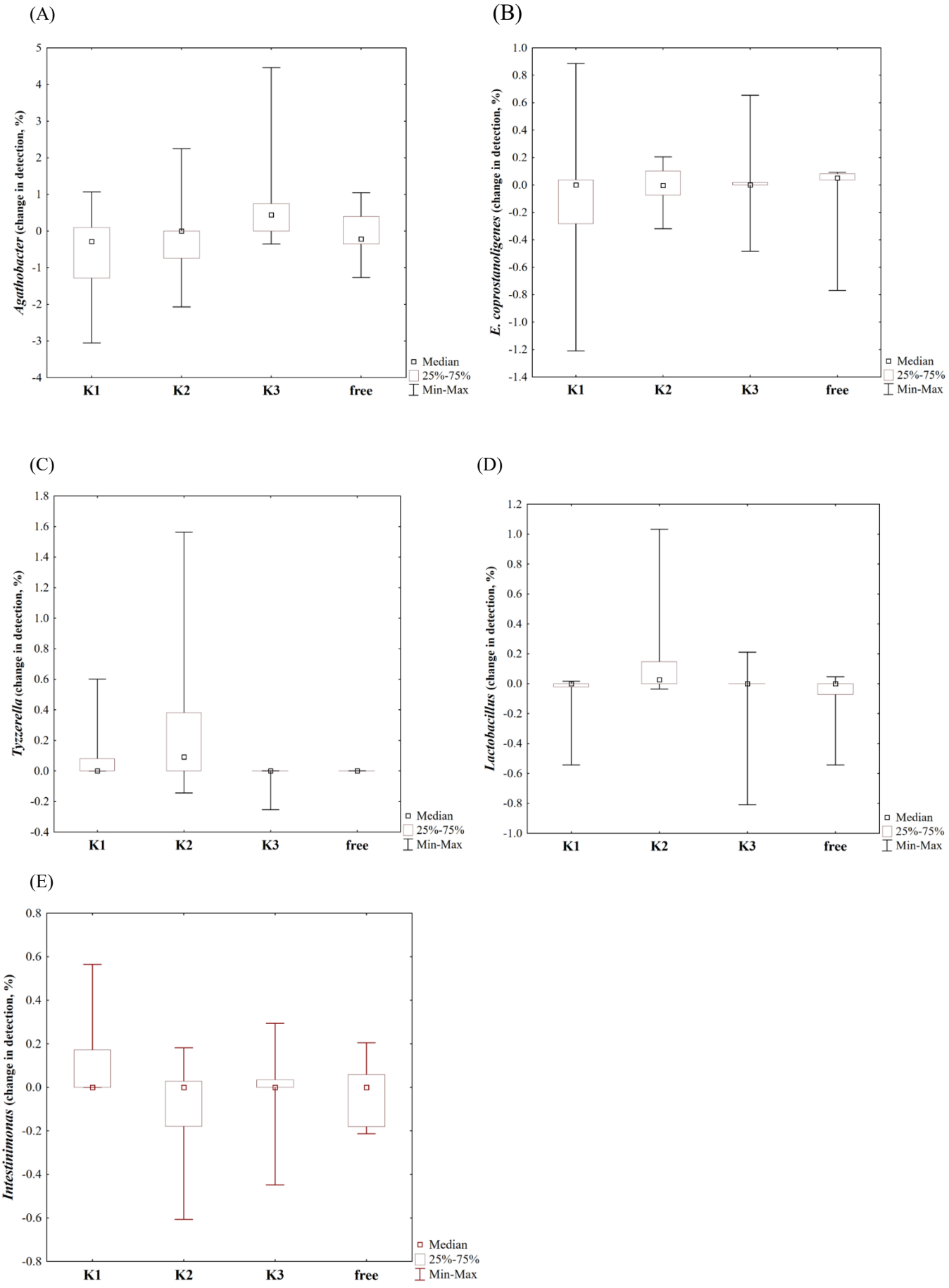


Fig. 4.16: Changes in the relative abundance (%) of *Agathobacter* spp. (A); *Eubacterium coprostanoligenes* spp. (B); *Tyzzerella* spp. (C); *Lactobacillus* (D) and *Intestinimonas* spp. (E).

The fact that GF plays a key role in shaping the celiac patients gut microbiota towards intestinal homeostasis is confirmed by the increase of two species, in the black and white zone, respectively, that contribute to the "healthy ecology" of the gut and to its homeostasis, by producing butyrate (Furusawa et al., 2013; Chen et al., 2018). These are the *Intestinimonas* and the *Agathobacter*. The latter uses acetate to produce butyrate (Rosero et al., 2016), instead, *Intestinimonas* has the peculiar ability to use aminoacids to grow, in particular, lysine and glutamate, producing butyrate (Bui et al., 2015). Again, the GF "new shape" could allow bacteria to digest it better, becoming, in this case, a substrate for the production of an important molecule such as the butyrate, involved in the gut homeostasis regulation. Butyrate is a well known SCFA, with trophic properties: it has long been known to be important as a carbon source for colonic epithelial cells (Roediger, 1982) and has been demonstrated its effect in regulating cTreg homeostasis (Smith et al., 2013). It enhances the intestinal epithelial barrier by regulating the expression and the assembly of TJs proteins and preventing their disruption (Rao et al., 2013; Krishnan et al., 2015; Aliberti, 2016). Furthermore, it modulates macrophage and DCs function, anti-inflammatory cytokine secretion, intestinal goblet cell mucin secretion and neutrophilchemotaxis (Sharma et al., 2010; Verdu et al., 2015; Blacher et al., 2017; Lin and Zhang, 2017).

The *Intestinimonas* increase, in the black zone, let us explain the lack of symptoms in these patients during the GF trial but also the uncoupling of some parameters such as the mucosal injury, the gut permeability, and cytokines. Although, not being as vigorous as Lactobacilli, *Intestinimonas* spp. have not been able, in three months, to immunomodulate IELs as it happened in the grey zone.

Surely, the time factor in the gut microbiota reshaping assumes an important role, and probably a longer study would have strengthened the results observed.

References

Agarwal, S., Kovilam, O., Zach, T.L., Agrawal, D.K. (2016). Immunopathogenesis and therapeutic approches in pediatric celiac disease. Expert Rev. Clinical Immunology, 12: 857-69. doi: 10.1586/1744666X.2016.1168294.

Aliberti, J. (2016). Immunity and Tolerance Induced by Intestinal Mucosal Dendritic Cells. Mediators of Inflammation; 2016:3104727. doi: 10.1155/2016/3104727.

Allin, K.H., Tremaroli, V., Caesar, R., Jensen, B.A.H., Damgaard, M.T.F., Bahl, M.I., Licht, T.R., Hansen, T.H., Nielsen, T., Dantoft, T.M., Linneberg, A., et al. (2018). Aberrant intestinal microbiota in individuals with prediabetes. Diabetologia; 61(4):810-820. doi: 10.1007/s00125-018-4550-1.

Arentz-Hansen, H., Korner, R., Molberg, O., Quarsten, H., Vader, W., Kooy, Y. M., et al. (2000). The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. J. Exp. Med. 191, 603–612. doi: 10.1084/jem.191.4.603.

Bevilacqua, A., Costabile, A., Bergillos-Meca, T., Gonzalez, I., Landriscina, L., Ciuffreda, E., D'Agnello, P., Corbo, M.R., Sinigaglia, M., Lamacchia, C. (2016). Impact of Gluten-Friendly Bread on the Metabolism and Function of In Vitro Gut Microbiota in Healthy Human and Coeliac Subjects. PLoS One; 1(9): e0162770. doi: 10.1371/journal.pone.0162770.

Blacher, E., Levy, M., Tatirovsky, E., Elinav, E. (2017). Microbiome-Modulated Metabolites at the Interface of Host Immunity. The Journal of Immunology; 198(2):572-580. doi: 10.4049/jimmunol.1601247.

Buckley, A., Turner, J.R. (2018). Cell Biology of Tight Junction Barrier Regulation and Mucosal Disease. Cold Spring Harb Perspect Biol. 2;10 (1). doi: 10.1101/cshperspect.a029314.

Bui, T.P., Ritari, J., Boeren, S., de Waard, P., Plugge, C.M., de Vos, W.M. (2015). Production of butyrate from lysine and the Amadori product fructoselysine by a human gut commensal. Nat. Commun.; 6:10062. doi: 10.1038/ncomms10062.

Burger, J., W., de Brouwer B., IntHout J., Wahab P.J., Tummers M., Drenth J.P.H. (2017). Systematic review with meta-analysis: Dietary adherence influences normalization of health-related quality of life in coeliac disease. Clin Nutr.; 36(2):399-406. doi: 10.1016/j.clnu.2016.04.021.

Caminero, A., Galipeau, H. J., McCarville, J. L., Johnston, C. W., Bernier, S. P., Russell, A.K., et al. (2016). Duodenal bacteria from patients with celiac disease and healthy subjects distinctly affect gluten breakdown and immunogenicity. Gastroenterology; 151, 670 –83. doi:10.1053/j.gastro.2016.06.041.

Caputo, I., Lepretti, M., Martucciello, S., Esposito, C. (2010). Enzymatic strategies to detoxify gluten: implications for celiac disease. Enzyme Res.; 2010:174354. doi: 10.4061/2010/174354.

Catassi, C., Fabiani, E., Iacono, G., D'Agate, C., Francavilla, R., Biagi, F., Volta, U., Accomando, S., Picarelli, A., De Vitis, I., Pianelli, G., Gesuita, R., Carle, F., Mandolesi, A., Bearzi, I., Fasano, A. (2007). A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. Am J Clin Nutr.; 85(1):160-6. doi: 10.1093/ajcn/85.1.160.

Chander, A.M., Yadav, H., Jain, S., Bhadada, S.K., Dhawan, D.K. (2018). Cross-Talk Between Gluten, Intestinal Microbiota and Intestinal Mucosa in Celiac Disease: Recent Advances and Basis of Autoimmunity. Front Microbiol.; 9:2597. doi: 10.3389/fmicb.2018.02597.

Chen, G., Ran, X., Li, B., Li, Y., He, D., Huang, B., Fu, S., Liu, J., Wang, W. (2018). Sodium Butyrate Inhibits Inflammation and Maintains Epithelium Barrier Integrity in a TNBS-induced Inflammatory Bowel Disease Mice Model. EBioMedicine; 30:317-325. doi: 10.1016/j.ebiom.2018.03.030.

Chen, L., Zou, Y., Peng, J., Lu, F., Yin, Y., Li, F., Yang, J. (2015). Lactobacillus acidophilus suppresses colitis-associated activation of the IL-23/Th17 axis. J. Immunol. Res.; 2015:909514. doi: 10.1155/2015/909514.

Coker, O.O., Dai, Z., Nie, Y., Zhao, G., Cao, L., Nakatsu, G., Wu, W.K., Wong, S.H., Chen, Z., Sung, J.J.Y., Yu, J. (2018). Mucosal microbiome dysbiosis in gastric carcinogenesis. Gut; 67(6):1024-1032. doi: 10.1136/gutjnl-2017-314281.

Costabile, A., Bergillos-Meca, T., Landriscina, L., Bevilacqua, A., Gonzalez-Salvador, I., Corbo, M.R., Petruzzi, L., Sinigaglia, M., Lamacchia, C. (2017). An In Vitro Fermentation Study on the Effects of Gluten FriendlyTM Bread on Microbiota and Short Chain Fatty Acids of Fecal Samples from Healthy and Celiac Subjects. Frontiers in Microbiology; 8:1722. doi: 10.3389/fmicb.2017.01722.

De Palma, G.; Nadal, I.; Medina, M.; Donat, E.; Ribes-Koninckx, C.; Calabuig, M.; Sanz, Y. (2010). Intestinal dysbiosis and reduced immunoglobulin-coated bacteria associated with coeliac disease in children. BMC Microbiol.10, 63.

De Vincenzi, M., Vincentini, O., Di Nardo, G., Boirivant, M., Gazza, L., Pogna, N., et al. (2010). Two prolamin peptides from durum wheat preclude celiac disease-specific T cell activation by gluten proteins. Eur. J. Nutr. 49, 251–255. doi: 10.1007/s00394-009-0080-4.
Dewar, D. H., Amato, M., Ellis, H. J., Pollock, E. L., Gonzalez-Cinca, N.,Wieser, H.,et al. (2006). The toxicity of high molecular weight glutenin subunits of wheat to patients with coeliac disease. Eur. J. Gastroenterol. Hepatol. 18, 483–491. doi: 10.1097/00042737-200605000-00005.
Fasano, A., Not, T., Wang, W., Uzzau, S., Berti, I, Tommasini, A., Goldblum, S.E. (2000). Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. Lancet, 355(9214):1518-9. doi: 10.1016/S0140-6736(00)02169-3.
Felli, C., Baldassarre, A., Masotti, A. (2017). Intestinal and Circulating MicroRNAs in Coeliac Disease. International Journal of Molecular Sciences, 18(9). doi: 10.3390/ijms18091907.
Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., Takahashi, M., Fukuda, N.N., Murakami, S., et al. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature; 504(7480):446-50. doi: 10.1038/nature12721.
Furusawa, Y., Obata, Y., Hase, K. (2015). Commensal microbiota regulates T cell fate decision in the gut. Seminars in immunopathology; 37(1):17-25. doi: 10.1007/s00281-014-0455-3.
Gervasoni, J., Primiano, A., Graziani, C., Scaldaferri, F., Gasbarrini, A., Urbani, A., Persichilli, S. (2018). Validation of UPLC-MS/MS Method for Determination of Urinary Lactulose/Mannitol. Molecules; 23(10).
Geuking, M.B., Cahenzli, J., Lawson, M.A., Ng, D.C., Slack, E., Hapfelmeier, S., McCoy, K.D., Macpherson, A.J. (2011). Intestinal bacterial colonization induces mutualistic regulatory T cell responses. Immunity; 34(5):794-806. doi: 10.1016/j.immuni.2011.03.021.
Girard-Madoux, M., Ober-Blöbaum, J., Costes, L., Kel, J.M., et al. (2016). IL-10 control of CD11c+ myeloid cells is essential to maintain immune homeostasis in the small and large intestine. Oncotarget, 7(22):32015-30. doi: 10.18632/oncotarget.8337.
Hansson, T., Dannaeus, A., Klareskog, L. (1999). Cytokine-producing cells in peripheral blood of children with coeliac disease secrete cytokines with a type 1 profile. Clinical & Experimental Immunology, 116(2):246-50. doi: 10.1046/j.1365-2249.1999.00882.x.
Heaton, K.W., Radvan, J., Cripps, H., Mountford, R.A., Braddon, F.E., Hughes, A.O. Defecation frequency and timing, and stool form in the general population: a prospective study. (1992). Gut; 33(6):818-24.
Hopper, A.D., Hadjivassiliou, M., Hurlstone, D.P., Lobo, A.J., McAlindon, M.E., Egner, W., Wild, G., Sanders, D.S. (2008). What is the role of serologic testing in celiac disease? A prospective, biopsy-confirmed study with economic analysis. Clin. Gastroenterol. Hepatol.; 6(3):314-20. doi: 10.1016/j.cgh.2007.12.008.
Iacomino, G., Marano, A., Stillitano, I., Aufiero, V.R., et al. (2016). Celiac disease: role of intestinal compartments in the mucosal immune response. Molecular and Cellular Biochemistry, 411(1-2):341-9. doi: 10.1007/s11010-015-2596-7.
Kelsall, B.L, Rescigno, M. (2004). Mucosal dendritic cells in immunity and inflammation. Nature Immunology, 5(11):1091-5. doi: 10.1038/ni1104-1091.
Khatkar, B. S. (2014). Structure and functionality of wheat gluten proteins. International Conference of Food Properties (ICFP).
Kim, K.S., Rowlinson, M.C., Bennion, R., Liu, C., Talan, D., Summanen, P., Finegold, S.M. (2010). Characterization of Slackia exigua isolated from human wound infections, including abscesses of intestinal origin. J Clin Microbiol.; 48(4):1070-5. doi: 10.1128/JCM.01576-09.
Krishnan, S., Alden, N., Lee, K. (2015). Pathways and functions of gut microbiota metabolism impacting host physiology. Current Opinion in Biotechnology; 36:137-45. doi: 10.1016/j.copbio.2015.08.015.
Lähdeaho, M.L., Kaukinen, K., Laurila, K., Vuotikka, P., Koivurova, O.P., Kärjä-Lahdensuu, T., Marcantonio, A., Adelman, D.C., Mäki, M. (2014). Glutenase ALV003 attenuates gluten-induced mucosal injury in patients with celiac disease. Gastroenterology; 146(7):1649-58. doi: 10.1053/j.gastro.2014.02.031.
Lähdeaho, M.L., Mäki, M., Laurila, K., Huhtala, H., Kaukinen, K. (2011). Small- bowel mucosal changes and antibody responses after low- and moderate-dose gluten challenge in celiac disease. BMC Gastroenterol.; 11:129. doi: 10.1186/1471-230X-11-129.
Lamacchia, C. (2016). Method for detoxification of gluten protein from grains of cereals and related medical uses Italian priority patent n°:102015000084813, Patent Cooperation Treaty PCT/EP2016/0815899.
Lamacchia, C., Di Luccia, A., & Gianfrani, C. (2015). Metodo per la detossificazione delle protein del glutine dalle granaglie dei cereali. Italian patent 0001414717. April 26th 2015.
Lamacchia, C., Di Luccia, A., and Gianfrani, C. (2013). Method for detoxification of gluten protein from grains of cereals. Patent Cooperation Treaty; PCT/IB2013/000797.

Lamacchia, C., Landriscina, L., & D'Agnello, P. (2016). Changes in wheat kernel proteins induced by microwave treatment. Food Chemistry, 197, 634–640.
Lamacchia, C., Musaico, D., Henderson, M. E., Bergillos-Meca, T., Roul, M., Landriscina, L., et al. (2018). Temperature-treated gluten proteins in Gluten-Friendly™ Bread increase mucus production and gut-barrier function in human intestinal goblet cells. J. Funct. Foods 48, 507–514. doi: 10.1016/j.jff.2018.07.047.
Landriscina, L., D'Agnello, P., Bevilacqua, A., Corbo, M. R., Sinigaglia, M., & Lamacchia, C. (2017). Impact of Gluten-Friendly™ technology on wheat kernel endosperm and gluten protein structure in seeds by light and electron microscopy. Food Chemistry, 221, 1258–1268.
Leffler, D., Schuppan, D., Pallav, K., Najarian, R., Goldsmith, J.D., Hansen, J., Kabbani, T., Dennis, M., Kelly, C.P. (2013). Kinetics of the histological, serological and symptomatic responses to gluten challenge in adults with coeliac disease. Gut; 62(7):996-1004. doi: 10.1136/gutjnl-2012-302196.
Leffler, D.A., Dennis, M., Edwards, George, J., Jamma, S., Cook, E.F., Schuppan, D., Kelly, C.P. (2009). A validated disease-specific symptom index for adults with celiac disease. Clinical Gastroenterology and Hepatology; 7(12):1328-34, 1334.e1-3.
Lin, L., Zhang, J. (2017). Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. BMC Immunol.; 18(1):2. doi: 10.1186/s12865-016-0187-3.
Ludvigsson, J.F., Ciacci, C., Green, P.H., Kaukinen, K., Korponay-Szabo, I.R., Kurppa, K., Murray, J.A., Lundin, K.E.A., Maki, M.J., Popp, A., Reilly, N.R., Rodriguez-Herrera, A., Sanders, D.S., Schuppan, D., Sleet, S., Taavela, J., Voorhees, K., Walker, M.M., Leffler, D.A. (2018). Outcome measures in coeliac disease trials: the Tampere recommendations. Gut; 67(8):1410-1424. doi: 10.1136/gutjnl-2017-314853.
Lv, W., Liu, C., Ye, C., Sun, J., Tan, X., Zhang, C., Qu, Q., Shi, D., Guo, S. (2017). Structural modulation of gut microbiota during alleviation of antibiotic-associated diarrhea with herbal formula. Int. J. Biol. Macromol.; 105(Pt 3):1622-1629. doi: 10.1016/j.ijbiomac.2017.02.060.
Mamone, G., Camarca, A., Fierro, O., Sidney, J., Mazzarella, G., Addeo, F., et al. (2013). Immunogenic peptides can be detected in whole gluten by transamidating highly susceptible glutamine residues: implication in the search for gluten-free cereals. J. Agric. Food Chem. 61, 747–754. doi: 10.1021/jf3040435.
Marasco, G.; Di Biase, A.R.; Schiumerini, R.; Eusebi, L.H.; Iughetti, L.; Ravaioli, F.; Scaioli, E.; Colecchia, A.; Festi, D. (2016). Gut microbiota and celiac disease. Dig. Dis. Sci. 61, 1461–1472.72.
Marsh, M.N., Crowe, P.T. (1995). Morphology of the mucosal lesion in gluten sensitivity. Baillieres Clin Gastroenterol.; 9(2):273-93. doi: 10.1016/0950-3528(95)90032-2.
Moheb-Alian, A., Forouzesh, F., Rostami-Nejad, M., Rostami, K. (2016). Mesenchymal stem cells as potential therapeutic approaches in celiac disease. Gastroenterology and Hepatology from Bed to Bench, 9 (Suppl1): S1-S7.
Molberg, O., Solheim Flaete, N., Jensen, T., Lundin, K. E., Arentz-Hansen, H., Anderson, O. D., et al. (2003). Intestinal T-cell responses to high-molecular weight glutenins in celiac disease. Gastroenterology 125, 337–344. doi: 10.1016/ S0016-5085(03)00890-4.
Mukhopadhya, I., Hansen, R., El-Omar, E.M., Hold, G.L. (2012). IBD-what role do Proteobacteria play? Nat Rev Gastroenterol Hepatol.; 9(4):219-30. doi: 10.1038/nrgastro.2012.14.
Mustalahti, K., Lohiniemi, S., Collin, P., Vuolteenaho, N., Laippala, P., Mäki, M. (2002). Gluten-free diet and quality of life in patients with screen-detected celiac disease. Eff Clin Pract.; 5(3):105-13.
Nadal, I., Donat, E., Ribes-Koninckx, C., Calabuig, M., Sanz, Y. (2007). Imbalance in the composition of the duodenal microbiota of children with coeliac disease. J. Med. Microbiol. 56, 1669–1674.
Niranjan, K.C., Tayaar, A., Kumar, G.S., Krishnapillai, R., Hallikeri, K., Hunasgi, S. (2016). Immunohistochemical Expression of Cyclin B1 in Epithelial Hyperplasia, Dysplasia and Oral Squamous Cell Carcinomas - A Comparative Study. J Clin Diagn Res.; 10(9):ZC85-ZC90. doi: 10.7860/JCDR/2016/19820.8563.
Ohue, R., Hashimoto, K., Nakamoto, M., Furukawa, Y., Masuda, T., Kitabatake, N., Tani, F. (2011). Bacterial heat shock protein 60, GroEL, can induce the conversion of naïve T cells into a CD4 CD25 (+) Foxp3-expressing phenotype. J Innate Immun.; 3(6):605-13. doi: 10.1159/000330786.
Paavola, A., Kurppa, K., Ukkola, A., Collin, P., Lähdeaho, M.L., Huhtala, H., Mäki, M., Kaukinen, K. (2012). Gastrointestinal symptoms and quality of life in screen-detected celiac disease. Dig Liver Dis.; 44(10):814-8. doi: 10.1016/j.dld.2012.04.019.
Pyle, G.G., Paaso, B., Anderson, B.E., Allen, D.D., Marti, T., Li, Q., Siegel, M., Khosla, C., Gray, G.M. (2005). Effect of pretreatment of food gluten with prolyl endopeptidase on gluten-induced malabsorption in celiac sprue. Clin. Gastroenterol. Hepatol.; 3(7):687-94. doi: 10.1016/s1542-3565(05)00366-6.

Qi, H., Li, Y., Yun, H., Zhang, T., Huang, Y., Zhou, J., Yan, H., Wei, J., Liu, Y., Zhang, Z., Gao, Y., Che, Y., Su, X., Zhu, D., Zhang, Y., Zhong, J., Yang, R. (2019). Lactobacillus maintains healthy gut mucosa by producing L-Ornithine. Commun. Biol.; 2:171. doi: 10.1038/s42003-019-0424-4.
Rao, R.K., Samak, G. (2013). Protection and Restitution of Gut Barrier by Probiotics: Nutritional and Clinical Implications. Current Nutrition & Food Science; 9(2):99-107. doi: 10.2174/1573401311309020004.
Rauhavirta, T., Qiao, S.W., Jiang, Z., Myrsky, E., Loponen, J., Korponay-Szabó, I.R., Salovaara, H., Garcia-Horsman, J.A., Venäläinen, J., Männistö, P.T., Collighan, R., Mongeot, A., Griffin, M., Mäki, M., Kaukinen, K., Lindfors, K. (2011). Epithelial transport and deamidation of gliadin peptides: a role for coeliac disease patient immunoglobulin A. Clin. Exp. Immunol.; 164(1):127-36. doi: 10.1111/j.1365-2249.2010.04317.x.
Reddel, S., Del Chierico, F., Quagliariello, A., Giancristoforo, S., Vernocchi, P., Russo, A., Fiocchi, A., Rossi, P., Putignani, L., El Hachem, M. (2019). Gut microbiota profile in children affected by atopic dermatitis and evaluation of intestinal persistence of a probiotic mixture. Sci Rep.; 9(1):4996. doi: 10.1038/s41598-019-41149-6.
Rescigno, M. (2008). Intestinal epithelial cells control dendritic cell function. Journal of Pediatric Gastroenterology and Nutrition, 46 Suppl 1: E17-9. doi: 10.1097/01.mpg.0000313831.09089.36.
Revicki, D.A., Wood, M., Wiklund, I., Crawley, J. (1998). Reliability and validity of the Gastrointestinal Symptom Rating Scale in patients with gastroesophageal reflux disease. Quality of Life Research; 7(1):75-83.
Revicki, D.A., Wood, M., Wiklund, I., Crawley, J. (1998). Reliability and validity of the Gastrointestinal Symptom Rating Scale in patients with gastroesophageal reflux disease. Quality of Life Research; 7(1):75-83. doi: 10.1023/a:1008841022998.
Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G.A.D., Gasbarrini, A., Mele, M.C. (2019). What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. Microorganisms; 7(1). pii: E14. doi: 10.3390/microorganisms7010014.
Rodriguez, B., Prioult, G., Hacini-Rachinel, F., Moine, D., Bruttin, A., Ngom-Bru, C., Labellie, C., Nicolis, I., Berger, B., Mercenier, A., Butel, M.J., Waligora-Dupriet, A.J. (2012). Infant gut microbiota is protective against cow's milk allergy in mice despite immature ileal T-cell response. FEMS Microbiol Ecol.; 79(1):192-202. doi: 10.1111/j.1574-6941.2011.01207.x.
Roediger, W.E. (1982). Utilization of nutrients by isolated epithelial cells of the rat colon. Gastroenterology; 83(2):424-9.
Rosero, J.A., Killer, J., Sechovcová, H., Mrázek, J., Benada, O., Fliegerová, K., Havlík, J., Kopečný, J. (2016). Reclassification of Eubacterium rectale (Hauduroy et al. 1937) Prévot 1938 in a new genus Agathobacter gen. nov. as Agathobacter rectalis comb. nov., and description of Agathobacter ruminis sp. nov., isolated from the rumen contents of sheep and cows. Int. J. Syst. Evol. Microbiol.; 66(2):768-773. doi: 10.1099/ijsem.0.000788.
Rostom, A., Murray, J.A., Kagnoff, M.F. (2006). American Gastroenterological Association (AGA) Institute technical review on the diagnosis and management of celiac disease. Gastroenterology; 131(6):1981-2002. doi: 10.1053/j.gastro.2006.10.004.
Round, J.L., Mazmanian, S.K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol.; 9(5):313-23. doi: 10.1038/nri2515.
Serena, G., Yan, S., Camhi, S., Patel, S., Lima, R. S., Sapone, A., et al. (2017). Proinflammatory cytokine interferon-gamma and microbiome-derivedmetabolites dictate epigenetic switch between forkhead box protein 3 isoformsin coeliac disease.Clin. Exp. Immunol.; 187, 490–506. doi: 10.1111/cei.12911.
Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G. M., et al. (2002). Structural basis for gluten intolerance in celiac sprue. Science 297, 2275–2279. doi: 10.1126/science.1074129.
Shan, L., Qiao, S.W., Arentz-Hansen, H., Molberg, Ø., Gray, G.M., Sollid, L.M., Khosla, C. (2005). Identification and analysis of multivalent proteolytically resistant peptides from gluten: implications for celiac sprue. J Proteome Res.; 4(5):1732-41. doi: 10.1021/pr050173t.
Sharma, R., Young, C., Neu, J. (2010). Molecular modulation of intestinal epithelial barrier: contribution of microbiota. Journal of Biomedicine and Biotechnology; 2010:305879. doi: 10.1155/2010/305879.
Shin, N.R., Whon, T.W., Bae, J.W. (2015). Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol.; 33(9):496-503. doi: 10.1016/j.tibtech.2015.06.011.
Siegel, M., Bethune, M. T., Gass, J., Ehren, J., Xia, J., Johannsen, A., et al. (2006). Rational design of combination enzyme therapy for celiac sprue. Chem. Biol. 13, 649–658. doi: 10.1016/j.chembiol.2006.04.009.
Silvester, J.A., Kurada, S., Szwajcer, A., Kelly, C.P., Leffler, D.A., Duerksen, D.R. (2017). Tests for Serum Transglutaminase and Endomysial Antibodies Do Not Detect Most Patients With Celiac Disease and Persistent Villous Atrophy on Gluten-free Diets: a Meta-analysis. Gastroenterology; 153(3):689-701.e1. doi: 10.1053/j.gastro.2017.05.015.

Smith, P.M., Howitt, M.R., Panikov, N., Michaud, M., Gallini, C.A., Bohlooly-Y, M., Glickman, J.N., Garrett, W.S. (2013). The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science; 341(6145):569-73. doi: 10.1126/science.1241165.
Sollid, L. M., and Khosla, C. (2005). Future therapeutic options for celiac disease. Nat. Clin. Pract. Gastroenterol. Hepatol. 2, 140–147. doi: 10.1038/ ncpgasthep0111.
Srinivas, M., Srinivasan, V., Jain, M. Rani Shanthi, C.S., Mohan, V., Jayanthi, V. (2019). A cross-sectional study of stool form (using Bristol stool chart) in an urban South Indian population. JGH Open; 3(6):464-467.
Taavela, J., Koskinen, O., Huhtala, H., Lähdeaho, M.L., Popp, A., Laurila, K., Collin, P., Kaukinen, K., Kurppa, K., Mäki, M. (2013). Validation of morphometric analyses of small-intestinal biopsy readouts in celiac disease. PLoS One; 11;8(10):e76163.
Takiishi, T., Fenero, C.I.M., Câmara, N.O.S. (2017). Intestinal barrier and gut microbiota: Shaping our immune responses throughout life. Tissue Barriers; 5(4):e1373208. doi: 10.1080/21688370.2017.1373208.
Tuominen, V.J. and Isola, J. (2010). Linking whole-slide microscope images with DICOM by using JPEG2000 interactive protocol. Journal of Digital Imaging; 23(4):454-62.
Tuominen, V.J., Ruotoistenmäki, S., Viitanen, A., Jumppanen, M., Isola, J. (2010). ImmunoRatio: a publicly available web application for quantitative image analysis of estrogen receptor (ER), progesterone receptor (PR), and Ki-67. Breast Cancer Research;12(4):R56.
Van de Wal, Y., Kooy, Y. M., van Veelen, P., Vader, W., August, S. A., Drijfhout, J. W., et al. (1999). Glutenin is involved in the gluten-driven mucosal T cell response. Eur. J. Immunol. 29, 3133–3139.
Vancamelbeke, M., Vermeire, S. (2017). The intestinal barrier: a fundamental role in health and disease. *Expert Review of Gastroenterology & Hepatology*, 11(9):821-834.
Verdu, E.F., Galipeau, H.J, Jabri, B. (2015). Novel player in coeliac disease pathogenesis: role of the gut microbiota. Nature Rev. Gastroenterology Hepatology; 12: 497-506. doi: 10.1038/nrgastro.2015.90.
Vorobjova, T., Tagoma, A., Oras, A., Alnek, K., Kisand, K. (2019). Celiac Disease in Children, Particularly with Accompanying Type 1 Diabetes, Is Characterized by Substantial Changes in the Blood Cytokine Balance, Which May Reflect Inflammatory Processes in the Small Intestinal Mucosa. Journal of Immunology Research, 2019:6179243. doi: 10.1155/2019/6179243.
Walker-Smith, J.A., Guandalini, S., Schmitz, J., Shmerling, D.H., Visakorpi, J.K. (1990). Revised criteria for diagnosis of coeliac disease. Arch. Dis. Child.; 65:909-11. doi:10.1136/adc.65.8.909.
Wang, L., Zhu, L., Qin, S. (2019). Gut Microbiota Modulation on Intestinal Mucosal Adaptive Immunity. J. Immunol. Res.; 2019:4735040. doi: 10.1155/2019/4735040.
Wieser, H. (2007). Chemistry of gluten proteins. Food Microbiol. 24, 115–119.
Zhang, Y., Hou, Q., Ma, C., Zhao, J., Xu, H., Li, W., Wang, Y., Ma, H., Zhang, H., Sun, Z. (2019). Lactobacillus casei protects dextran sodium sulfate- or rapamycin-induced colonic inflammation in the mouse. Eur. J. Nutr. doi: 10.1007/s00394-019-02001-9.

Chapter 5

General Discussion

Gluten Friendly™ technology is a patented [1, 2], scientific temperature-based method that reshapes the gluten proteins inside cereal kernels, before milling. The application of high temperature for a short amount of time to hydrated wheat kernels induces significant structural changes completely different from those observed in a gluten model system and pasta [3]. Previous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) protein profiles suggested the aggregation between proteins of the same class. This could be possible because gluten is not yet formed in the cells of the starchy endosperm of the kernel, but gluten proteins are segregated, in their native form, into different protein bodies that accumulate simultaneously and independently in the wheat endosperm [5]. Furthermore, the SDS-PAGE analysis of protein fractions under reducing and non-reducing conditions showed that microwave treatment caused neither a decrease in the number of the bands nor a decrease in their intensity [4]. Thus, protein denaturation did not occur. Lamacchia et al. [4] suggested that the brief high temperatures generated by microwaves applied to the hydrated caryopses led to conformational changes in the gluten proteins, as evidenced by the increase in total cysteine levels. Thus, it has been postulated that Gluten Friendly™ technology allows the reshaping of the tertiary structure of gluten proteins by breaking the hydrogen bonds between glutamine residues when proteins are in their native form into protein bodies, promoting chemical changes otherwise impossible in the already formed gluten. Among the structural modifications of wheat proteins, the gluten solubility in saline aqueous solutions and the *in vitro* reduced immunogenicity of the most common epitopes involved in CD [4] are the most noteworthy. Moreover, previous *in vitro* studies conducted on wheat kernels treated with the Gluten Friendly™ technology showed a reduced cross-reactivity of antibodies recognizing almost the entire range of gluten proteins [6]. This leads to the conformational changes of the gluten proteins in the kernel. Although the temperature-treated gluten shows reduced immunogenic properties *in vitro*, it does not lose its technological properties and viscoelasticity needed to process flour into bread, pasta, and other baked goods [4]. Moreover, the organoleptic and nutritional qualities of Gluten Friendly bread (GFB) do not differ significantly from those of the control bread (CB). Additional *in vitro* researches [7,8] found that GFB was able to positively modify the quali-quantitative composition of gut microbiota (in terms of Lactobacilli and Bifidobacteria members) in both celiac and healthy individuals, maintaining the intestinal microbial balance. The GFB was also able to boot the production of Short Chain Fatty Acids (SCFAs) by probiotics and contrast the pathogenic agents such as *S. Typhimurium* and *S. aureus.* It should be pointed out that the composition and diversity of gut microbiota play a pivotal role in gut homeostasis, providing beneficial effects to the host [9]. Scientific evidence highlight that intestinal dysbiosis leads to impairment of oral tolerance and it seems to be associated with CD onset or development [10]. Moreover, SCFAs (butyrate, acetate, propionate) released by microbiota also have an important role.

They act as key sources of energy for colorectal tissues, promote cellular mechanisms that maintain tissue integrity, prevent inflammation and pathogen invasion, and help to regulate ions absorption [11, 12]. Based on these *in vitro* results, the effects of the GFB on mucus production and gut barrier function were here investigated. The cell culture experiments were carried out using mucus-secreting intestinal goblet cells (HT29-16E) which represent a healthy human model with a high predictive value to study mucus secretion *in vitro.* The mucus layer is the first line of physical and biochemical defense towards external molecules and bacteria that could reach the epithelial cell layer [13]. Furthermore, the thickness and formulation of the mucus vary along the different segments of the gut, [14] and normal mucus production is indicative of a healthy gut epithelium whereas a reduced mucus production has been found in pathologic conditions such as CD [15, 16]. In this thesis, mucus production was examined using both Periodic acid–Schiff (PAS) and Alcian-blue (AB) staining techniques [17, 18]. A positive PAS reaction indicates the presence of neutral mucins. A positive Alcian Blue reaction at pH 1.0 and 2.5 indicates the presence of acidic sulfated and acidic carboxylated mucins, respectively. The transmembrane mucin MUC3, a main component of the enterocyte glycocalyx, stains positive for PAS and can be considered as neutral mucin. Conversely, MUC2, the major gel-forming mucin of the outer mucus layer, stains positive for AB and can be considered as acidic mucin [19, 20]. The data presented in this thesis demonstrated that GFB did not affect cell viability, as indicated by the MTT assay, but increased the secretion of mucins as observed after PAS and AB staining, in comparison with the control cells. To better investigate the effect of GFB on mucin secretion, specific MUC2 and MUC3 Human enzyme-linked immunosorbent assays (ELISA) were carried out to quantify the levels of these two mucins in the supernatants and cell lysates, respectively. MUC2 ELISA results supported the finding that GFB induced higher production of MUC2 than the control and CB. Instead, no significant difference was observed in MUC3 secretion throughout different experiment conditions. Despite of the MUC2 is critical for the formation of the intestinal mucus barrier, it also provides food for commensal bacteria, prevents pathogens from reaching the glycocalyx beneath, and significantly affects the immunogenicity of the gut antigens by delivering tolerogenic signals to dendritic cells, via the intestinal epithelial cells. Therefore, taking into account these proprieties, it can be assumed that MUC2 enhances gut homeostasis [21-24]. A reduced MUC2 secretion has indeed been associated with a range of gastrointestinal inflammatory diseases [25, 26]. The data presented here support the potential of GFB to maintain and/or enhance gut homeostasis and intestinal barrier function, by maintaining higher mucin levels. The mode by which GFB stimulates mucin production requires further investigation. However, results collected so far suggest that exposure of hydrated wheat caryopses to the Gluten Friendly™ method induces a different spatial conformation of the amino acid sequences and a reshaping of the tertiary structure of

gluten proteins. It has been postulated that such rearrangement of the gluten protein structure could expose positive charges, namely cationic residues, that could also be acting as mucin secretagogues on HT29-16 E mucus-secreting cells. Indeed, mucus secretion is triggered by a wide array of bioactive factors, including neuropeptides, microbial products, cytokines, reactive oxigen and nitrogen species [70]. Furthermore, the integrity of the cells monolayer before and after 24h treatment with the digested bread was evaluated by the Trans Epithelial Electrical Resistance (TEER) measurements. The integrity of the monolayer was confirmed by TEER > 200Ω/cm^2. In spite of some limitations of the *in vitro* cell model [27], the data showed here indicate the potential for GFB to improve the intestinal cellular barrier integrity, as indicated by the significantly higher TEER increase, compared to both control and CB. It is worth to underline that an increased TEER signal indicates improved intestinal barrier function. It could be assumed that the increased trans-epithelial electrical resistance is also due to the increase in the MUC2 secretion, even though the glycocalyx and tight junction proteins are also key contributors [28].

There is now scientific consensus regarding the importance of the gut microbiota in health and disease, and there are many factors that influence its composition [9, 29]. It has been reported that the microbiota may modulate intestinal goblet cell mucin secretion [10, 30, 31]. Several data show that the gut microbiota determines the properties of the mucus gel, whilst the constitution of the mucus layer can also affect the microbiota functions [32-34]. Thus, there is a mutual interaction between the mucus and the gut microbiota. Furthermore, previous studies showed that GFB positively modulated the complex ecosystem of gut microbiota with an increase in numbers of health-promoting beneficial bacteria in both healthy and celiac patients [7, 8]. Therefore, taken together with the data showed here, the intestinal epithelium mucus production may be increased not only by a direct effect of GFB itself but also as a consequence of gut microbiota modulation triggered by GFB. In the final analysis, the GFB has the potential to induce mucin secretion by goblet cells, improve intestinal epithelial barrier function, and contribute to the gut homeostasis.

Furthermore, since the results of the mentioned study [7] highlighted a protective effect of GFB on *L. acidophilus* viability with a significant lowering of its death rate and a prolongation of the survival time, another analysis on the impact of the Gluten Friendly Flour (GFF) on the functionality of an active drink was carried out. In particular, the study was based on the idea to design a synbiotic fermented milk containing GFF as a beneficial ingredient and *L. acidophilus* as functional starter. During the design of a synbiotic food, it is mainly important that the prebiotic component must not affect the performances of the starter microorganism. Thus, the first phase of this research consisted in the assessment of the acidification of *L. acidophilus* in presence of flour and as a function of the level of inoculum and temperature in order to optimize the conditions to produce the active drink.

The fermented milk was produced by inoculating *L. acidophilus* at 6.5 log cfu/ml, adding flour at 2.5 g/l and performing the fermentation at 37°C. During the refrigerated storage of the synbiotic drink produced, the GFF showed a positive effect on the death kinetic of the probiotic with a significant prolongation of the shoulder length to 20 days (12-13 days in the control or in presence of control flour). As reported in the literature, prebiotics could act toward lactobacilli through three possible ways: effect on metabolism, induction of higher stability of membrane and starvation. For instance, fructooligosaccharides (FOSs) can affect the regulation of genes involved in the metabolism of fatty acids, proteins and cell wall [35]. FOSs and inulin can also stabilize the membrane against some stresses [36] or can induce a kind of starvation which can lead to an increase in viability as a practical output [37]. The data collected on GFF suggest that it cannot be simply labeled as a prebiotic, but it acts quite differently, lowering the death rate. The mechanism of action of GFF is not completely understood, but it could reside in its technology. Indeed, it has been postulated that Gluten Friendly™ technology reshapes the tertiary structure of gluten proteins, allowing the exposure of the positive charges, which in turn could interact with the teichoic acids on the cell wall of lactobacilli. This interaction could protect the cell from aging, and/or change the membrane permeability [7]. Another idea is a possible use of the modified gluten as an alternative source of energy for lactobacilli under stressful conditions. Probably, the right mode of action may consist of a combination of a positive effect on permeability and the use as a nutrient. However, further investigations are needed in order to clarify the molecular mechanism of action. Nevertheless, the novelty of the study here reported is a structured statistical approach on the quantitative effects of GFF on the survival of *L. acidophilus* La-5 and represents the first step to set up and design synbiotic fermented milk, combining this probiotic and GFF. Moreover, it is also worth underlining that the use of GFF did not act on the sensory scores and on the physico-chemical parameters of the active milk. Thus, the GFF could be considered a beneficial ingredient with a dual effect both on the viability of the probiotic, prolonging the shelf-life of the product, and on the quali-quantitative composition of the gut microbiota as previously demonstrated. In the final analysis, the outcomes reported in this thesis clearly show the bioactive properties of the Gluten Friendly™ molecule and give the possibility to obtain functional flours with beneficial qualities for digestive health and function.

Based on *in vitro* promising outcomes which have demonstrated a reduced immunoreactivity of Gluten Friendly™ molecule on celiac, gut-deriver T-cells, a lower cross-reactivity with R5 and other antibodies against gluten, and its bioactive proprieties, an *in vivo* prospective, double-blind, randomized, placebo-controlled study was carried out. The trial of 12 weeks was performed on 48 celiac volunteers to evaluate the efficacy and safety of the prolonged ingestion of GFB in patients with celiac disease already on a gluten-free diet. Several analyses were carried out throughout the 12

weeks of daily intake of GFB on duodenal biopsies, urine and stool samples, symptoms and serological samples of celiac volunteers.

The CD is characterized by a variety of histological lesions and symptoms attributable to gluten intake. Typically, the inflammation triggered by gluten promotes partial to total villus atrophy, crypt hyperplasia and the infiltration of IELs (Intraepithelial Lymphocytes) in the gut mucosa [38, 39]. An increase in IELs count indicates ongoing inflammation. After 12 weeks on a Gluten Friendly diet, K-means clustered the celiac subjects in three zones: 35% black zone (cluster 1, K1), 30% grey zone (cluster 2, K2), 35% white zone (trend as the placebo group) (cluster 3, K3). The statistic pinpointed an uncoupling among some parameters. Namely, Vh/CrD significantly decreased in black and grey zone, but surprisingly it was not related to IELs increase in the grey zone and to the detection of 33-mer in K1 and 2 (black and grey zones). This suggests a positive effect in 35% of patients (white zone) and a possible dynamic situation, with a positive modulation of the inflammation, in 30% of patients (grey zone). Besides, subjects in all three zones showed symptoms relief and a not significant increase of inflammatory cytokines and gut permeability. This response to celiac subjects, so peculiar, to the administration of GF, has never been observed in the gluten challenge study, which on the contrary, at even lower doses than those used in the GF study, caused a significant Vh/CrD decrease coupling with an IELs increase together with a symptoms, cytokines, and gut permeability increase [40, 41]. Furthermore, in any study, that has used 33-mer antibody to identify gluten in stools after its intake, absence has never occurred; par excellence, in fact, gluten is a mixture of highly indigestible molecules [42, 43].

Several researchers stressed the importance of microbiota in celiac disease [44] and reported that celiac subjects generally show typical microbial imprinting with some prevailing genera/family (Gram negative, pro-inflammatory microbiota). As a preliminary result, the microbiota of stool at baseline was analyzed to check if the subjects of the trials were homogeneous or if a difference in the initial microbiota could be responsible for the different outcome to Gluten Friendly™ bread.

The presence of two pro-inflammatory genera such as *Slackia* and *Sutterella* in celiac subjects of the grey and black zones, concerning the white zone, had a key role in the response to GF administration. Indeed, *Slackia* and *Sutterella* jointly contributed to the pro-inflammatory response observed [45-49]. It is well known that the environmental infectious agents compete with the beneficial microbes to adhere to the intestinal mucosa, and after adherence, they disturb intestinal barrier function (tight junctions) by activating different inflammatory pathways at the intestinal mucosal surface [50]. Thus, a disturbed intestinal barrier leads to exposure of intestinal immune cells to the dietary antigens (i.e., gluten).

The presence of *Slackia* and *Sutterella* allowed to explain the mucosal injury in the grey and black zones, but it did not explain the 33-mer absence in the white zone and such a low detection percentage in the grey and black zones. It also did not explain why the intestinal injury was not correlated with the inflammation in the grey zone, and how symptoms relief was possible in areas with intestinal damage as well as a significant increase in gut permeability and inflammatory cytokines was not identified.

The absence of 33-mer in the stool of the white zone has been interpreted in only one way. The GF was completely digested. This would have been possible for two reasons: i) Gluten Friendly is a new type of gluten that has a "new shape" that probably allows the microbiota endopeptidases to digest it all; ii) the white zone did not present pro-inflammatory genera that could also "badly" cut the GF making it immunotoxic.

Specifically, new studies further highlighted the value of gut microbes in the determination of gluten immunogenicity, namely the fact that pathobionts isolated from CD patients cleaved 33-mer peptide in such a manner that it activated gluten-specific T-cells in CD patients [51].

By virtue of this, the presence of *Slackia* and *Sutterella* in K1 and 2 (black and grey zones) did not allow to all celiac subjects (47% and 36% respectively), to digest completely and correctly “Gluten Friendly™”. Furthermore, they predisposed intestine to inflammation [50] so that, even traces (under 40 ppm; not detectable by G-12 immunoassay) of 33-mer, maybe present in 53% and 64% of patients in the black and grey zones (K1 and 2) respectively, became detrimental for the gut. This would explain the uncoupling parameters in K1 and 2 about histological injury and presence/absence of 33-mer, but could also explain the different sensitivity of celiac patients to a very low amount of gluten [52].

The non-correlation between intestinal damage and inflammation in the grey zone (K2), and the improvement of symptoms in the two zones that experienced the intestinal injury, underline positive changes of microbiota as a result of GF supplementation. Each zone experienced a significant increase in butyrate-producing positive species.

In particular, increased *Lactobacillus* in the grey zone elucidates why the intestinal damage (Vh/CrD) at the end of the three months trial was uncoupled with inflammation (IEL), and solve the question of the relief of symptoms in the celiac patients at least in this zone. *Lactobacillus* is a well-known genus with various beneficial properties. It can contribute to gut re-equilibration and shape the microbiota towards homeostasis, with a protective effect against some diseases [53, 54]. Moreover, *Lactobacillu*s might promote the homeostasis of the gut mucus layer through producing L-Orn. L-Orn stimulates Trp metabolism to produce AhR ligands in gut epithelial cells, which induce accumulation of RORγt (+) IL-22(+) ILC3 in gut tissues [55].

Their significant increase observed in the grey zone is undoubtedly attributable to the administration of Gluten Friendly, since the GF had already shown, *in vitro*, [7, 8] an effect on the growth of Lactobacilli and their metabolism (bifidogenic effect).

It could be supposed that although in this zone the microbiota was not in homeostasis, the GF, correctly digested, was used as a source of energy to Lactobacilli that were turning out the inflammation and may be carrying out gut microbiota towards homeostasis and immunological tolerance. Indeed, evidence suggests that gut commensal, (including *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Clostridium*, and *Streptococcus)*, as well as bacterial metabolites, such as butyric and propionic acid, may program Treg cells in the intestine towards tolerogenicity [56-59], and help the host with maturation and homeostasis of immune system [30].

The fact that GF plays a key role in shaping the celiac patients gut microbiota towards intestinal homeostasis is confirmed by the increase of two species, in the black and white zone, respectively, that contribute to the "healthy ecology" of the gut and to its homeostasis, by producing butyrate [60, 61]. These are the *Intestinimonas* and the *Agathobacter*: the latter uses acetate to produce butyrate [62], instead, *Intestinimonas* has the peculiar ability to use aminoacids to grow, in particular, lysine and glutamate, producing butyrate [63]. Again, the GF "new shape" could allow bacteria to digest it better becoming a substrate for the production of an important molecule such as the butyrate, involved in the gut homeostasis regulation.

Butyrate is a well-known SCFA, with trophic properties: it has long been known to be important as a carbon source for colonic epithelial cells [64] and has been demonstrated its effect in regulating cTreg homeostasis [65]. It enhances the intestinal epithelial barrier by regulating the expression, the assembly of TJs proteins, and preventing their disruption [66-68]. Furthermore, it modulates macrophage and DCs function, anti-inflammatory cytokine secretion, intestinal goblet cell mucin secretion and neutrophilchemotaxis [10, 30, 31, 69].

The increased *Intestinimonas*, in the black zone, let us explain the lack of symptoms in these patients during the GF trial but also the uncoupling of some parameters such as the mucosal injury, the gut permeability, and cytokines. Although, not being as vigorous as *Lactobacillus*, *Intestininomonas* spp. have not been able, in three months, to immunomodulate IELs as it happened in the grey zone.

Surely, the time factor in the gut microbiota reshaping assumes an important role, and probably a longer study would have strengthened the results observed.

Conclusion

This thesis is aimed to understand the biochemical mechanisms induced by the tertiary structure modification of the Gluten Friendly™ molecule *in vitro* and *in vivo.*

The Gluten Friendly™ technology is an innovative temperature-based process that reshapes gluten proteins inside cereal kernels, before milling. The antigenic property of gluten is abolished and the *in vitro* immunogenicity of the most common epitopes of gluten is reduced. This method keeps all the nutritional, organoleptic and rheological proprieties of cereals. Gluten Friendly ™ flour leavens into bread and pizza and stretches into all pasta shapes. The technology turns gluten into a bioactive protein. Flour becomes a functional ingredient, with beneficial qualities for digestive health. In particular, previous studies confirmed that GFB strengthened the gut microbiota of celiac and healthy subjects *in vitro,* after 48 hours and 30 days respectively. It boosted SCFAs production by Lactobacilli and Bifidobacteria members and contrasted pathogenic agents. Furthermore, the results showed in this thesis highlighted that digested GFB stimulated *in vitro* mucus secretion and also strengthened intestinal barrier function, enhancing gut homeostasis. In synbiotic fermented milk, the GFF exerted a positive effect on the viability of the probiotic *L. acidophilus,* with a prolongation of the shoulder length to 20 days. The GFB was also tested through a prospective, double-blind, randomized, placebo-controlled *in vivo* study on 48 celiac volunteers. The results of the study suggested that, after 12 weeks of daily intake, the GFB had the potential of: i) improving gluten-related symptoms over time; ii) to be completely digested by the gut microbiota in absence of pro-inflammatory species; iii) reshaping gut microbiota towards gut homeostasis. The mechanism of action of Gluten Friendly molecule™ is may be due to its “new shape” that has become easily digestible by gut microbiota endopeptidase and that use it as a source of energy. GF allows some good species, such as *Agathobacter*, *Lactobacillus*, and *Intestinimonas* to proliferate and produce metabolites, such as butyric and propionic acid that may program Treg cells in the intestine towards tolerogenicity, and help the host with maturation and homeostasis of the immune system.

References

1. Lamacchia, C., Di Luccia, A., and Gianfrani, C. (2013). Method for detoxification of gluten protein from grains of cereals. Patent Cooperation Treaty; PCT/IB2013/000797.

2. Lamacchia, C. (2016). Method for detoxification of gluten protein from grains of cereals and related medical uses; Italian priority patent n°:102015000084813, Patent Cooperation Treaty PCT/EP2016/0815899.

3. Lamacchia C., Baiano, A., Lamparelli, S., La Notte, E., Di Luccia, A. (2010). Changes in durum wheat kernel and pasta proteins induced by toasting and drying processes. *Food Chemestry*, 118, 191-198.

4. Lamacchia, C., Landriscina, L., & D'Agnello, P. (2016). Changes in wheat kernel proteins induced by microwave treatment. *Food Chemistry*, 197, 634–640.

5. Tosi, P., Gritsch, C.S., He, J., Shewry, P.R. (2011). Distribution of gluten proteins in bread wheat (Triticum aestivum) grain. *Annals of Botany,* 108(1):23-35.

6. Landriscina, L., D'Agnello, P., Bevilacqua, A., Corbo, M. R., Sinigaglia, M., & Lamacchia, C. (2017). Impact of gluten-friendly™ technology on wheat kernel endosperm and gluten protein structure in seeds by light and electron microscopy. *Food Chemistry*, 221, 1258–1268.

7. **Bevilacqua, A.**, Costabile, A., Bergillos-Meca, T., Gonzalez, I., Landriscina, L., Ciuffreda, E., D'Agnello, P., Corbo, M.R., Sinigaglia, M., Lamacchia, C. (2016). Impact of Gluten-Friendly Bread on the Metabolism and Function of In Vitro Gut Microbiota in Healthy Human and Coeliac Subjects. *PLoS One*, 1(9): e0162770.

8. Costabile, A., Bergillos-Meca, T., Landriscina, L., Bevilacqua, A., Gonzalez-Salvador, I., Corbo, M.R., Petruzzi, L., Sinigaglia, M., Lamacchia, C. (2017). An In Vitro Fermentation Study on the Effects of Gluten Friendly™ Bread on Microbiota and Short Chain Fatty Acids of Fecal Samples from Healthy and Celiac Subjects. *Frontiers in Microbiology*, 8:1722.

9. Marasco, G., Di Biase, A.R., Schiumerini, R., Eusebi, L.H., Iughetti, L., Ravaioli, F., Scaioli, E., Colecchia, A., Festi, D. (2016). Gut Microbiota and Celiac Disease. *Digestive Diseases and Sciences*, 61(6):1461-72.

10. Verdu, E.F., Galipeau, H.J, Jabri, B. (2015). Novel player in coeliac disease pathogenesis: role of the gut microbiota. Nature Rev. *Gastroenterology Hepatology*, 12: 497-506.

11. Tan, J., McKenzie, C., Potamitis, M., Thorburn, A.N., Mackay, C.R., Macia, L. (2014). The role of short-chain fatty acids in health and disease. *Advances in Immunology*, 121:91-119.

12. Ohira, H., Tsutsui, W., Fujioka, Y. (2017). Are Short Chain Fatty Acids in Gut Microbiota Defensive Players for Inflammation and Atherosclerosis? *Journal of Atherosclerosis and Thrombosis*, 24(7):660-672.

13. **Turner, J. R.** (2009). Intestinal mucosal barrier function in health and disease. *Nature Review and Immunology*, 9, 799–809.

14. **Rescigno, M.** (2017). The microbiota revolution: Excitement and caution. *European Journal of Immunology*, 47(9):1406-1413.

15. Forstner, G. (1995). Signal transduction, packaging and secretion of mucins. *Annual Review of Physiology*, 57, 585–605.

16. Bischoff, S. C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J. D., Serino, M.,Wells, J. M. (2014). Intestinal permeability – A new target for disease prevention and therapy. *BMC Gastroenterology*, 14, 189.

17. Diebel, L. N., Liberati, D. M., & Hall-Zimmerman, L. (2011). H2 blockers decrease gut mucus production and lead to barrier dysfunction in vitro. *Surgery*, 150, 736–743.

18. Steedman, H. F. (1950). Alcian blue 8GS: A new stain for mucin. *The Quarterly Journal of Microscopical Science*, 91, 477–479.

19. **Pelaseyed, T.**, Bergström, J.H., Gustafsson, J.K., Ermund, A., Birchenough, G.M., Schütte, A., Van der Post, S., Svensson, F., Rodríguez-Piñeiro, A.M., Nyström, E.E., Wising, C., Johansson, M.E., Hansson, G.C. (2014). The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunological Reviews*, 260(1):8-20.

20. Kim, Y. S., & Ho, S. B. (2010). Intestinal goblet cells and mucins in health and disease: Recent insights and progress. *Current Gastroenterology Reports*, 12, 319–330.

21. Arike, L., & Hansson, G. C. (2004). The densely O-glycosylated MUC2 mucin protects the intestine and provides food for the commensal bacteria. *Journal of Molecular Biology*, 428, 3221–3229.

22. Allaire, J.M., Morampudi, V., Crowley, S.M., Stahl, M., Yu, H., Bhullar, K., Knodler, L.A., Bressler, B., Jacobson, K., Vallance, B.A. (2018). Frontline defenders: goblet cell mediators dictate host-microbe interactions in the intestinal tract during health and disease. *Am J Physiol Gastrointest Liver Physiol.* 314(3): G360-G377.

23. Johansson, M.E., Hansson, G.C. (2016). Immunological aspects of intestinal mucus and mucins. *Nature Reviews Immunology*, 16(10):639-49.

24. **Shan, M.,** Gentile, M., Yeiser, J.R., Walland, A.C., et al. (2013). Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. *Science*, 342(6157):447-53.
25. Cobo, E.R., Kissoon-Singh, V., Moreau, F., Chadee, K. (2015). Colonic MUC2 mucin regulates the expression and antimicrobial activity of β-defensin 2. *Mucosal Immunology*, 8(6):1360-72.
26. Makkink, M. K., Schwerbrock, N. M., Mähler, M., Boshuizen, J. A., Renes, I. B., Cornberg, M., Dekker, J. (2002). Fate of goblet cells in experimental colitis. *Digestive Diseases and Sciences*, 47, 2286–2297.
27. Martínez-Maqueda, D., Miralles, B. & Recio, I. (2015). HT29 cell line. In K. Verhoeckx et al. (Eds.), The impact of food bio-actives on gut health (pp. 117–118).
28. Lee, S. H. (2015). Intestinal permeability regulation by tight junction: Implication on inflammatory bowel diseases. *Intestinal Research*, 13, 11 –18.
29. Ulluwishewa, D., Anderson, R. C., McNabb, W. C., Moughan, P. J., Wells, J. M., & Roy, N. C. (2011). Regulation of tight junction permeability by intestinal bacteria and dietary components. *The Journal of Nutrition*, 141, 769–776.
30. Lin, L., Zhang, J. (2017). Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. *BMC Immunology*, 6;18(1):2.
31. Sharma, R., Young, C., Neu, J. (2010). Molecular modulation of intestinal epithelial barrier: contribution of microbiota. *Journal of Biomedicine and Biotechnology*, 2010:305879.
32. Vancamelbeke, M., Vermeire, S. (2017). The intestinal barrier: a fundamental role in health and disease. *Expert Review of Gastroenterology & Hepatology*, 11(9):821-834.
33. Jakobsson, H.E., Rodríguez-Piñeiro, A.M., Schütte, A., et al. (2015). The composition of the gut microbiota shapes the colon mucus barrier. *EMBO Reports*,16(2):164–177.
34. Johansson, M.E., Hansson, G.C. (2016). Immunological aspects of intestinal mucus and mucins. *Nature Reviews Immunology*, 16(10):639-49.
35. Saulnier, D., Spinler, J. K., Gibson, G. R., and Versalovic, J. (2009). Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods. *Current Opinion in Biotechnology*, 20,135–14.
36. Schwab, C., Vogel, R., and Ganzle, M. G. (2007). Infuence of oligosaccharides on the viability and membrane properties of Lactobacillus reuteri TMW1.106 during freeze-drying. *Cryobiology* 55, 108–114.
37. Altieri, C., Bevilacqua, A., and Sinigaglia, M. (2011). Prolonging the viability of Lactobacillus plantarum through the addition of prebiotics into the medium. *Journal of Food Science*, 76, M336–M345.
38. Sapone, A., Bai, J.C., Ciacci, C., Dolinsek, J., Green, P.H., Hadjivassiliou, M., Kaukinen, K., Rostami, K., Sanders, D.S., Schumann, M., Ullrich, R., Villalta, D., Volta, U., Catassi, C., Fasano, A. (2012). Spectrum of gluten-related disorders: consensus on new nomenclature and classification. *BMC Medicine*, 7; 10:13.
39. Hossein-Nataj, H., Masjedi, M., Emami, M.H., Mokhtari, M., Alsahebfosoul, F. (2019). Cell Density Counts of the Intestinal Intraepithelial Lymphocytes in the Celiac Patients. *Iranian Journal of Immunology*, 16(2):117-126.
40. Leffler, D., Schuppan, D., Pallav, K., Najarian, R., Goldsmith, J.D., Hansen, J., Kabbani, T., Dennis, M., Kelly, C.P. (2013). Kinetics of the histological, serological and symptomatic responses to gluten challenge in adults with coeliac disease. *Gut*; 62(7):996-1004.
41. Leffler, D.A., Dennis, M., Edwards, George, J., Jamma, S., Cook, E.F., Schuppan, D., Kelly, C.P. (2009). A validated disease-specific symptom index for adults with celiac disease. *Clinical Gastroenterology and Hepatology*; 7(12):1328-34, 1334.e1-3.
42. Mamone, G., Camarca, A., Fierro, O., Sidney, J., Mazzarella, G., Addeo, F., et al. (2013). Immunogenic peptides can be detected in whole gluten by transamidating highly susceptible glutamine residues: implication in the search for gluten-free cereals. *J. Agric. Food Chem*. 61, 747–754.
43. Wieser, H. (2007). Chemistry of gluten proteins. Food Microbiol. 24, 115–119.
44. Chander, A.M., Yadav, H., Jain, S., Bhadada, S.K., Dhawan, D.K. (2018). Cross-Talk Between Gluten, Intestinal Microbiota and Intestinal Mucosa in Celiac Disease: Recent Advances and Basis of Autoimmunity. *Front Microbiol.;* 9:2597.
45. Reddel, S., Del Chierico, F., Quagliariello, A., Giancristoforo, S., Vernocchi, P., Russo, A., Fiocchi, A., Rossi, P., Putignani, L., El Hachem, M. (2019). Gut microbiota profile in children affected by atopic dermatitis and evaluation of intestinal persistence of a probiotic mixture. *Sci Rep.*; 9(1):4996.
46. Allin, K.H., Tremaroli, V., Caesar, R., Jensen, B.A.H., Damgaard, M.T.F., Bahl, M.I., Licht, T.R., Hansen, T.H., Nielsen, T., Dantoft, T.M., Linneberg, A., et al. (2018). Aberrant intestinal microbiota in individuals with prediabetes. *Diabetologia*; 61(4):810-820.

47. Lv, W., Liu, C., Ye, C., Sun, J., Tan, X., Zhang, C., Qu, Q., Shi, D., Guo, S. (2017). Structural modulation of gut microbiota during alleviation of antibiotic-associated diarrhea with herbal formula. *Int. J. Biol. Macromol.*; 105(Pt 3):1622-1629.
48. Kim, K.S., Rowlinson, M.C., Bennion, R., Liu, C., Talan, D., Summanen, P., Finegold, S.M. (2010). Characterization of Slackia exigua isolated from human wound infections, including abscesses of intestinal origin. *J Clin Microbiol*.; 48(4):1070-5.
49. Coker, O.O., Dai, Z., Nie, Y., Zhao, G., Cao, L., Nakatsu, G., Wu, W.K., Wong, S.H., Chen, Z., Sung, J.J.Y., Yu, J. (2018). Mucosal microbiome dysbiosis in gastric carcinogenesis. *Gut*; 67(6):1024-1032.
50. Serena, G., Yan, S., Camhi, S., Patel, S., Lima, R. S., Sapone, A., et al. (2017). Proinflammatory cytokine interferon-gamma and microbiome-derivedmetabolites dictate epigenetic switch between forkhead box protein 3 isoformsin coeliac disease.*Clin. Exp. Immunol*.; 187, 490–506.
51. Caminero, A., Galipeau, H. J., McCarville, J. L., Johnston, C. W., Bernier, S. P., Russell, A.K., et al. (2016). Duodenal bacteria from patients with celiac disease and healthy subjects distinctly affect gluten breakdown and immunogenicity. *Gastroenterology*; 151, 670 –83.
52. Catassi, C., Fabiani, E., Iacono, G., D'Agate, C., Francavilla, R., Biagi, F., Volta, U., Accomando, S., Picarelli, A., De Vitis, I., Pianelli, G., Gesuita, R., Carle, F., Mandolesi, A., Bearzi, I., Fasano, A. (2007). A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. *Am J Clin Nutr.*; 85(1):160-6.
53. Chen, L., Zou, Y., Peng, J., Lu, F., Yin, Y., Li, F., Yang, J. (2015). Lactobacillus acidophilus suppresses colitis-associated activation of the IL-23/Th17 axis. *J. Immunol. Res.*; 2015:909514.
54. **Zhang, Y.**, Hou, Q., Ma, C., Zhao, J., Xu, H., Li, W., Wang, Y., Ma, H., Zhang, H., Sun, Z. (2019). Lactobacillus casei protects dextran sodium sulfate- or rapamycin-induced colonic inflammation in the mouse. *Eur. J. Nutr*. doi: 10.1007/s00394-019-02001-9.
55. **Qi, H**., Li, Y., Yun, H., Zhang, T., Huang, Y., Zhou, J., Yan, H., Wei, J., Liu, Y., Zhang, Z., Gao, Y., Che, Y., Su, X., Zhu, D., Zhang, Y., Zhong, J., Yang, R. (2019). Lactobacillus maintains healthy gut mucosa by producing L-Ornithine. *Commun. Biol*.; 2:171.
56. Geuking, M.B., Cahenzli, J., Lawson, M.A., Ng, D.C., Slack, E., Hapfelmeier, S., McCoy, K.D., Macpherson, A.J. (2011). Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity*; 34(5):794-806.
57. Ohue, R., Hashimoto, K., Nakamoto, M., Furukawa, Y., Masuda, T., Kitabatake, N., Tani, F. (2011). Bacterial heat shock protein 60, GroEL, can induce the conversion of naïve T cells into a CD4 CD25 (+) Foxp3-expressing phenotype. *J Innate Immun.*; 3(6):605-13.
58. Rodriguez, B., Prioult, G., Hacini-Rachinel, F., Moine, D., Bruttin, A., Ngom-Bru, C., Labellie, C., Nicolis, I., Berger, B., Mercenier, A., Butel, M.J., Waligora-Dupriet, A.J. (2012). Infant gut microbiota is protective against cow's milk allergy in mice despite immature ileal T-cell response. *FEMS Microbiol Ecol*.; 79(1):192-202.
59. Furusawa, Y., Obata, Y., Hase, K. (2015). Commensal microbiota regulates T cell fate decision in the gut. *Seminars in immunopathology*; 37(1):17-25.
60. Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., Takahashi, M., Fukuda, N.N., Murakami, S., et al. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*; 504(7480):446-50.
61. Chen, G., Ran, X., Li, B., Li, Y., He, D., Huang, B., Fu, S., Liu, J., Wang, W. (2018). Sodium Butyrate Inhibits Inflammation and Maintains Epithelium Barrier Integrity in a TNBS-induced Inflammatory Bowel Disease Mice Model. *EBioMedicine*; 30:317-325.
62. Rosero, J.A., Killer, J., Sechovcová, H., Mrázek, J., Benada, O., Fliegerová, K., Havlík, J., Kopečný, J. (2016). Reclassification of Eubacterium rectale (Hauduroy et al. 1937) Prévot 1938 in a new genus Agathobacter gen. nov. as Agathobacter rectalis comb. nov., and description of Agathobacter ruminis sp. nov., isolated from the rumen contents of sheep and cows. Int. J. Syst. Evol. *Microbiol.*; 66(2):768-773.
63. Bui, T.P., Ritari, J., Boeren, S., de Waard, P., Plugge, C.M., de Vos, W.M. (2015). Production of butyrate from lysine and the Amadori product fructoselysine by a human gut commensal. *Nat. Commun*.; 6:10062.
64. Roediger, W.E. (1982). Utilization of nutrients by isolated epithelial cells of the rat colon. *Gastroenterology*; 83(2):424-9.

65. Smith, P.M., Howitt, M.R., Panikov, N., Michaud, M., Gallini, C.A., Bohlooly-Y, M., Glickman, J.N., Garrett, W.S. (2013). The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science;* 341(6145):569-73.
66. Rao, R.K., Samak, G. (2013). Protection and Restitution of Gut Barrier by Probiotics: Nutritional and Clinical Implications. *Current Nutrition & Food Science*; 9(2):99-107.
67. Krishnan, S., Alden, N., Lee, K. (2015). Pathways and functions of gut microbiota metabolism impacting host physiology. *Current Opinion in Biotechnology*; 36:137-45.
68. Aliberti, J. (2016). Immunity and Tolerance Induced by Intestinal Mucosal Dendritic Cells. *Mediators of Inflammation*; 2016:3104727.
69. Blacher, E., Levy, M., Tatirovsky, E., Elinav, E. (2017). Microbiome-Modulated Metabolites at the Interface of Host Immunity. *The Journal of Immunology*; 198(2):572-580.
70. Kim, Y. S., & Ho, S. B. (2010). Intestinal goblet cells and mucins in health and disease: Recent insights and progress. Current Gastroenterology Reports, 12, 319–330.

List of publications

Lamacchia, C., **Musaico, D.**, Henderson, Mary E., Bergillos-Meca, T., Roul, M., Landriscina, L., Decina, I., Corona, G., Costabile, A. (2018). Temperature-treated gluten proteins in Gluten-Friendly™ bread increase mucus production and gut-barrier function in human intestinal goblet cells. *Journal of Functional Foods* 48, 507-514.

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Daniela Musaico
University of Foggia, Italy
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