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Department of the Sciences of Agriculture, Food and Environment (SAFE)

Doctoral Thesis

in

Management of Innovation in Agricultural and Food Systems of the Mediterranean Region

Study of Chemical, Biochemical and Technological characteristics of *Gluten Friendly*^{TM} grains of cereals and derived products

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Abstract

This thesis aimed to investigate chemical, biochemical and technological characteristics of *Gluten Friendly™* grains of cereals and derived products.

"*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 to obtain gluten detoxified flours suitable for the preparation of bakery products and pasta made from wheat. The "*Gluten Friendly™*" technology implies 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 aiming to combine the nutritional and technological properties of wheat proteins with safety for coeliac sufferers and other gluten-sensitive subjects. This innovation relies in a wise combination of temperatures with other parameters (moisturizing, evaporation, resting, etc.,) that are clearly described in the patent and non-patent literature quoted in this thesis and does not cause proteins to be denatured but a change in their configuration that makes them not recognizable by specific antibodies and able to produce further pleiotropic effects on man. In particular, "*Gluten Friendly™*" technology causes a rearrangement of the secondary and tertiary structure of the gluten proteins, involving a different spatial conformation of the sequences, including the so-called antigenic. So the antigenic capacity of gluten it is abolished and the immunogenicity in vitro of the most common epitopes involved in coeliac disease is reduces. Moreover, proteins structure rearrangements induced by "*Gluten Friendly™*" 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 visible at the immunofluorescent microscopy. Although, the rearrangement of the secondary and tertiary structure and the exposure of charges determines the solubility of proteins in "Gluten Friendly™" flours increasing the electrostatic repulsion and breaking the hydrogen the flours are able to form dough and leaven and produce bread. The sensory qualities of "Gluten Friendly™" bread is comparable to that of the Control Bread in terms of appearance, taste, aroma, color and bread texture. Furthermore, experiments carried out in vitro on healthy and coeliac human faecal microbiota pinpointed that "Gluten Friendly™" bread prolonged the survival of L.acidophilus and exerted an antibacterial effect towards S.aureusand S. Typhimurium. Moreover, "Gluten Friendly™" bread modulated the intestinal microbiota in vitro, promoting changes in lactobacilli and bifidobacteria members in coeliac subjects. A final multivariate approach combining both viable counts and metabolites

suggested that "Gluten Friendly™" bread could beneficially modulate the coeliac gut microbiome. These results were confirmed by an in vitro study performed by a three-stage continuous fermentative system on the intestinal microbiota and metabolites composition of healthy and coeliac individuals for 30 days.

Aim and outline of the thesis

This thesis is aimed on the study of chemical, biochemical and technological characteristics of *Gluten Friendly™* grains of cereals and derived products. Following the general introduction in *Chapter 1*, *Chapter 2* describes the effect of "Gluten Friendly™" (GF) technology on flour protein fractions, by size exclusion high-performance liquid chromatography (SE-HPLC), sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and R5-sandwich ELISA. *Chapter 3* describes the impact of Gluten-Friendly™ technology (Italian priority patent n_ 102015000084813 filed on 17th December 2015) on wheat kernel endosperm morphology and gluten protein structure, using SEM, light and immunofluorescent microscopy. Microscopy was combined with immunodetection with specific antibodies for gliadins, c-gliadins, LMW subunits and antigenic epitopes to gain a better understanding of the technology at a molecular level. In *Chapter 4* the chemical, rheological and pasting properties of Gluten Friendly Flours was investigated. In this study, attempt was made to evaluate technological proterties of Gluten Friendly Flours in the production of bread by analyzing the rheological properties of the flour and dough, as well as baking qualities and sensorial evaluation of the resulting bread.

Chapter 5 describes the *in vitro* response of healthy and coeliac human faecal microbiota to gluten-friendly bread (GFB). Thus, GFB and control bread (CB) were fermented with faecal microbiota in pH-controlled batch cultures. The effects on the major groups of microbiota were monitored over 48 h incubations by fluorescence *in situ* hybridisation. Short-chain fatty acids (SCFAs) were measured by high-performance liquid chromatography (HPLC). Furthermore, the death kinetics of *Lactobacillus acidophilus*, *Bifidobacterium animalis* subsp. *Lactis*, *Staphilococcus aureus*, and *Salmonella* Typhimurium in a saline solution supplemented with GFB or CB were also assessed. *Chapter 6* aimed to investigate the *in vitro* effects of GF bread (GFB) and control bread on the intestinal microbiota and metabolites composition of healthy and coeliac individuals in a validated three-stage continuous fermentative system simulating the proximal, transverse and distal parts of human colon (vessel 1, 2 and 3, respectively),

resemble the complexity and diversity of the intestinal microbiota. Main bacterial groups of the faecal microbiota were evaluated using 16S rRNA-based fluorescence *in situ* hybridization (FISH). Potential effects on microbial metabolism were also studied measuring short chain fatty acids (SCFAs) by HPLC analysis.

To finalize, *Chapter 7* is the general discussion on the obtained results of the patented method, conclusions and future works.

Chapter 1

General Introduction

1. Wheat

Cereals constitute the most important source of food worldwide and cereal products form the basis of the human diet in most countries. Cereal crops are also an important source of nutrition for animals and, moreover, they have many other important economic and ecological features (production of oils, starch, flour, sugar, malt and alcoholic beverages, renewable energy, etc.) [1, 2]. Cereals such as maize, rice, wheat, sorghum, millet, barley and oats are particularly important as a staple food in many countries, but wheat stands out from the others. Wheat is the world`s leading cereal and wheat grains are the major source of energy and proteins in human nutrition. Although wheat is grown on more land area than any other crop, it is only the third cereal crop in total world production, behind maize and rice [1]. Nevertheless, wheat is the most widely cultivated food crop for several reasons. Wheat is well adapted to harsh environments and, therefore, it can be grown in wide range of elevations, climatic conditions and soil fertility [3, 4]. It can be safely stored for a long period, easily transported and processed into various types of food. Wheat has high content of gluten proteins (wheat storage proteins) enabling to form a strong, cohesive dough that retain gas and produce light baked products. On that account, most breads, even rye and oat bread, are made with a portion of wheat flour. Wheat was one of the first domesticated cereals originated in the Middle East, area that stretches from Israel and Lebanon into Syria, Turkey, Iran and Iraq [1, 5]. Cultivation and repeated harvesting and sowing of wild grass grains led to the selection of mutant forms, which was the important part of wheat domestication. The cultivation started about 11,000 years ago, in the Middle East and by 5,000 years ago, wheat was cultivated from England to China [1]. In the 1600`s the wheat was introduced in America by Spaniards. Development of reaping and threshing machines in 1800`s (steam engine) and 1900`s (internal combustion engine) resulted in increased farmer productivity and wheat field became larger [1].

Wheat belongs to the grass family Ponceau, the tribe Triticale and the genus Tritium. The genus Tritium comprise several species and subspecies, some of which are cultivated and some are uncultivated, or rarely so. However, the botanical classification of wheat varieties is not unified. The most economically important species is Tritium ostium also known as common or bread wheat [1, 6]. This species, which is mainly used for baked products such as bread, covers up to 95 % of the wheat grown today. Remaining 5 % is mostly durum wheat (Tritium durum), traditionally used for pasta products [3, 7]. Other wheat species (e.g., spelt wheat) are grown in limited quantities, mostly for specialized use. Within a species, wheat varieties can be further classified by planting season (winter vs. spring wheat), hardness of the grain (hard vs. soft wheat), and the color of the grain (red, white or amber) [1, 8]. 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. 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. Spring wheat is significantly less yielding than the winter wheat but it usually offers very high quality bread making wheat. Hard wheat grains have higher gluten content than soft wheat varieties. The hardest wheat is durum, whose flour is used for macaroni, spaghetti and other pasta products. The soft wheat grains have higher starch-to-protein ratio compared to hard wheat varieties [1]. In general, hard wheat flour is used for bread and soft wheat flour for pastry. Nevertheless, many flours are carefully blended mixtures of both hard and soft wheat varieties designed for certain purpose.

Wheat grain (also known as a seed or kernel) is the most economically important part of the wheat plant, because it constitutes main source of people food. The grain is, in fact, the fruit of the wheat, botanically known as caryopsis [2]. It consists of three distinct parts: bran, endosperm and germ (Fig. 1). Bran, the outer coat consisting of seven cell layers, is separated from the flour during most milling processes. Bran surrounds germ and the endosperm and protects the grain from weather, insects, soil, and bacteria. It accounts for about 14 % of the grain weight [9]. Bran has low protein content but is rich in indigestible cellulose material called dietary fibers. It also contains B-vitamins and trace minerals. Bran is included in whole-wheat flour. The endosperm is the largest and most important part of the grain comprising about 83 % of the total grain mass, and consists mostly of starch (about 75 % of the endosperm mass). The endosperm contains the greatest proportion of carbohydrates (in starch granules), proteins, iron and B-vitamins. The starchy endosperm is the only material of wheat grain, from which the white flour is made [4, 9, 10]. Germ is the embryo or sprouting section of the seed comprising about 2.5 % of the grain weight and is usually separated from the flour during most milling processes because of fat content (about 10 %) that limits the flour quality. Fats can easily go rancid, which limits shelf life of the flour. Germ is present in whole-wheat flour. Besides lipids, germ further contains minimal quantities of proteins, and a greater share of B-vitamins and trace minerals [9].

1.2 Wheat grain proteins

Amongst the cereals, wheat is unique because wheat flour has the ability to form dough suitable for production of bread and wide range of baked products. Wheat flour is also widely used in starch and gluten industry, etc. Bread making quality is determined, in particular, by protein composition of the wheat grain. Within the cereal group, wheat has higher protein content than rice or corn. The mature wheat grains contain 8-20 % proteins, which are mostly present in the endosperm [4]. Although some of these proteins are important structural or metabolic proteins, majority of wheat grain proteins

(gluten proteins) are storage proteins that function as a source of nitrogen during the grain germination.

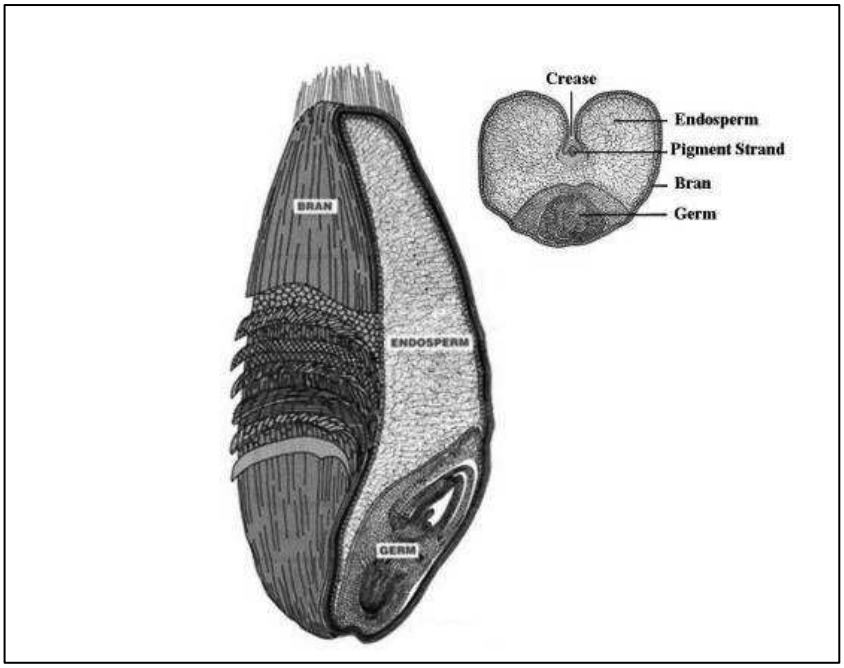

Fig. 1. Schematic diagram of the wheat grain illustrating its major parts.

Cereal proteins located in the endosperm constitute a heterogeneous group of proteins. At the beginning of the 20th Century, T. B. Osborne classified these proteins into four groups upon their solubility in a series of solvents [11]. The following protein fractions are subsequently extracted from the milled grain: albumins, water-soluble proteins; globulins, soluble in diluted salt solutions.; prolamins, soluble in aqueous alcohols (wheat prolamins are called gliadins); glutelin's, soluble in dilute alkali or acid (wheat glutelin's are called glutenin's). This classification is still in use despite the number of factors, such as fineness of grain milling, vigor and time of shaking, concentration of extraction solutions used, and the extraction temperature, which influencing the extractability of particular protein fraction. Moreover, each of mentioned fractions is a complex mixture of different proteins, which overlap in their solubility. This is particularly observed in gliadin and glutenin fractions. Thus, all four fractions can be contaminated by any proteins from other fractions [12, 13]. Recently, the wheat grain proteins are more often classified based on biological characteristics of the proteins together with their chemical and genetic relationships. Nevertheless, the Osborne`s classification system was a major milestone in the development of cereal chemistry. Water-soluble albumins and salt-soluble globulins constitute up to 20 % of total wheat grain proteins. These proteins are involved, particularly, 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 [12, 14]. Albumins and globulins can also play a role

as nutrient reserves for germinating embryo. They are, in contrast to gliadins and glutenin's, rich in essential amino acids, mainly lysine [12, 15]. Due to the amino acid composition both these protein groups are important with respect to nutritional requirements of humans and monastic animals as well. On the other hand, albumins and globulins are minority fractions of wheat grain proteins and, therefore, the wheat grain does not provide as high amount of nutritionally indispensable amino acids as do animal protein-based foods [12, 16]. However, the wheat grain proteins are complementary to other food proteins (e.g. animal products, legume) in terms of nutritional value. Molecular weight (MW) pattern of albumins and globulins is ranging from 2,000 to 106,000 and is predominantly divided into two relatively wide regions with MW from 23,000 to 66,000 and 2,000-16,000 [13]. In general, albumins and globulins have lower MW than gliadins and glutenin's. Among the varieties both albumins and globulins show very similar protein pattern and, consequently, these proteins are not suitable for wheat varieties identification [13]. The most important protein fractions of wheat grain, in many respects, are gliadins and glutenin's, known under general name gluten proteins or gluten, eventually. The investigation of wheat grain proteins, their relationships, metabolism and structures is an important research area, because these proteins impact the flour and nutritional quality [14]. There are several promising studies and approaches (including proteomics and genomics) focused to improvement of both grain yield and the end-use quality of wheat [3-5, 10, 14, 17-21].

1.3 Gluten proteins

Gluten is water-insoluble, viscoelastic and cohesive mass that remains after removal starch and water-soluble components from the wheat dough by water. Gluten proteins, conventionally subdivided into gliadins and glutenin's, are the major storage proteins of the wheat grain that make up about 80 % of total wheat grain proteins [4, 14, 22]. Both gliadins and glutenin's play a key role in dough rheology and bread making quality. In dough, gliadins are responsible for viscosity and extensibility whereas glutenin's are cohesive and distinctively affect the elasticity (dough strength). Thus, qualitative and quantitative constitution of gluten proteins is a crucial factor for dough formation and baking properties [25-27]. This is one of the major reasons for which the gluten proteins are the most investigated proteins of wheat grain. While gliadins are monomeric proteins (single polypeptide chains), glutenin's are present as oligo- and polymers (multiple polypeptide chains), where the glutenin subunits are linked 19 together by interchain desulphated bonds [10, 12, 28]. Gluten proteins are unique in terms of their amino acid composition. They have extremely high content of glutamine and proline and, on the contrary, low content of essential amino acids such as lysine. Another very important amino acid (besides the proline and glutamine) is cysteine, because it determines the structure and functionality of gluten [29]. Cysteines are mostly present in oxidized state and form

either intrachain desulphated bonds within a single protein (both gliadins and glutenin's) or interchain desulphated bonds between proteins (glutenin's) [28]. It has been found that number of these bonds increase in heated gluten [30]. Both intra- and interchain desulphated bonds, as well as non-covalent (hydrogen, hydrophobic) interactions are involved in the formation of gluten polymer complex. Gluten proteins are classically divided into monomeric gliadins and polymeric glutenin's. However, after reduction of desulphated bonds individual glutenin subunits are soluble in aqueous alcohols as well as gliadins. For this reason, both gliadins and glutenin's can be classified as prolamins [10, 12, 17, 40]. More recently, based on the structural and evolutionary relationships, the gluten proteins are divided into three broad groups (Fig. 2). Sulphur-poor prolamins include the ω-gliadins, the LMW-GS and α/β- and γ- gliadins belong to the Sulphur-rich prolamins and the HMW prolamins include the HMW-GS. From the biological point of view the role of gluten proteins is to provide a store of carbon, nitrogen and Sulphur to support grain germination and seedling growth. Furthermore, these proteins determine bread making quality of the wheat. Finally, gluten proteins are also known as an environmental factor triggering celiac disease (CD) in genetically-predisposed individuals [43, 44]. Both fractions are important contributors to the rheological properties of dough, though their functions are divergent. Hydrated gliadins have little elasticity and are less cohesive than glutenin's, and contribute mainly to the viscosity and extensibility of the dough. In contrast, hydrated glutenin's are both cohesive and elastic and are responsible for dough strength and elasticity [24,25]. A proper mixture of both fractions is essential for the quality of the end product. However, of particular importance are the glutenin polymers, and it is well established that strong (*i.e.*, highly viscos-elastic) doughs contain high proportions of HMW glutenin polymers [24]. Numerous studies are consistent with the hypothesis that the HMW subunits form an elastomeric polymer network which provides a "backbone" for interactions with other glutenin subunits and with gliadins (Figure 3) [26,27]. There is no doubt that this network is mainly stabilized by inter-chain desulphated bonds [28,29]. Additional covalent bonds formed during dough making are tyrosine-tyrosine and they-tyrosine crosslinks between gluten proteins [30,31,32]. However, the covalent structure of the gluten network is superimposed by non-covalent bonds (hydrogen bonds, ionic bonds, hydrophobic bonds) [33].

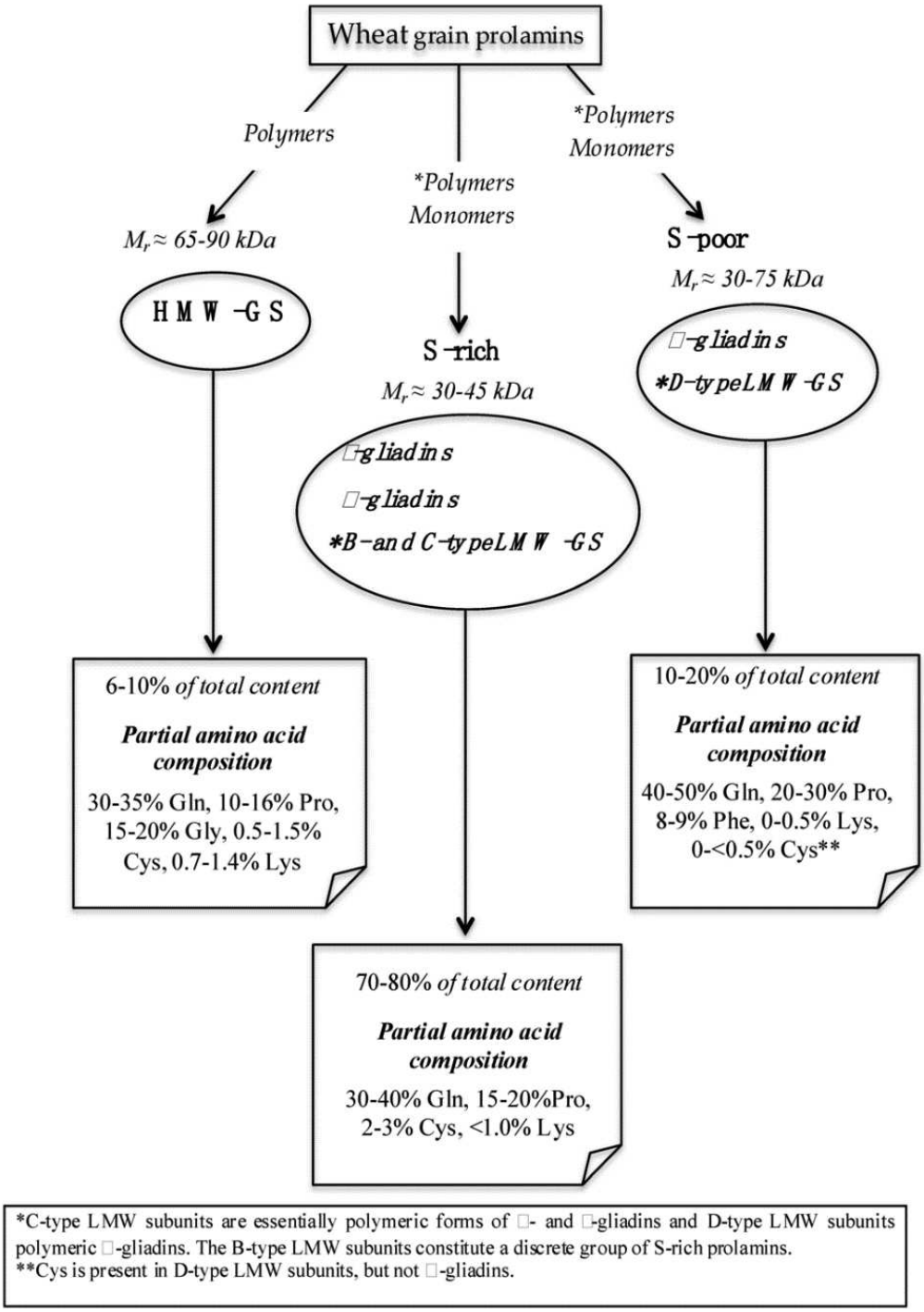

Figure 2. Schematic representation of types and peculiarities of wheat gluten proteins

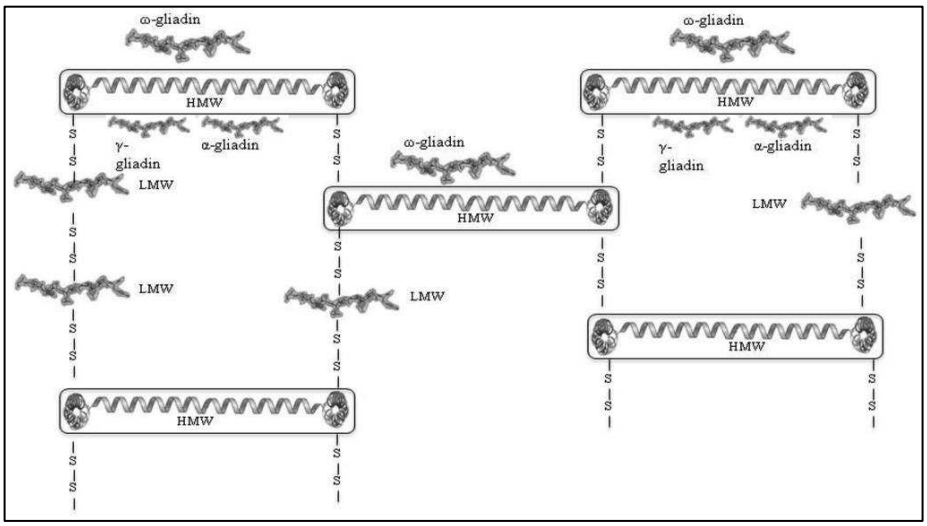

Figure 3. A structural model for wheat gluten in which the HMW subunits provide a disulphide-bonded backbone that interacts with other gluten proteins by disulphide bonds (LMW subunits) and non-covalent interactions (gliadins).

1.4 Gliadins

Gliadins are basically present as monomers and they are classically divided into four groups upon their mobility at low pH in gel electrophoresis (GE): α-, β-, γ- and ω-gliadins (in order of decreasing mobility) [12, 28, 31]. However, electrophoretic mobility of particular gliadin subgroups does not correspond to amino acid composition of these proteins. The α- and β- gliadins are closely related and, consequently, they fall into one group, α/β-type. On that account the gliadins are usually divided into three major groups: α/β-, γ- and ω-gliadins. Gliadins can be also classified in accordance to their amino acid composition and MW into four different types: α/β-, γ-, ω1,2- and ω5-gliadins [28, 32] (Figure 4). The ω-gliadins are minor components of gliadin fraction and they distinctively differ from other gliadins. The ω-gliadins lack cysteine (so there is no possibility to form desulphated bonds) and they are also characterized by higher proportions of glutamine, proline and phenylalanine compared to other gliadins [12, 28, 32, 33]. These three amino acids account approximately 80 % of the total amino acid composition. Based on different amino acid composition and MW, the ω-gliadins are further categorized into ω1,2- and ω5-gliadins. Within ω-gliadins, the ω5-type have higher contents of glutamine and phenylalanine and higher MW than ω1,2-type. The MWs of ω-gliadins are between 40,000 and 75,000. The primary sequence of these proteins consists almost entirely of octapeptide repetitive motifs (e.g., PQQPFPQQ) rich in glutamine and proline [12, 28, 31]. The α/β- and γ-gliadins have lower MWs that are overlapping (MW ranging from 28,000 to 40,000). In addition, they have lower proportion of glutamine and proline than ω-gliadins. On the other hand, the content of glutamine and proline remains high. The α/β- and γ-gliadins are relatively high in leucine and tyrosine compared to ω-gliadins [12, 28]. Each of these gliadin subgroups has different N- and C-

terminal domains. N-terminal domain is largely formed by repetitive sequences, and is unique for each type. With respect to C-terminal domain, α/β- and γ-gliadins are homologous. Most of α/β-gliadins contain six and most of γ- gliadins eight cysteine residues. As a result, they form three and four intrachain desulphated bonds, respectively [12, 28, 29]. Some gliadins have an odd number of cysteine residues due to point mutations [28]. Those gliadins are linked together or to glutenin's and form oligomers with MW ranging from around 100,000-500,000. Such oligomers are called high molecular weight (HMW) gliadins. These complexes are solely formed by α/β-gliadins, γ-gliadins and low molecular weight (LMW) glutenin's [28]. Substitution, insertion or deletion of amino acid residues are the common events occurring in each gliadin type [28, 34]. Although the gliadin composition is characteristic to variety and growing conditions, it can be generalized that α/β- and γ-gliadins occur in much higher proportions than ω-gliadins. The gliadin proteins are coded by genes located on the short arms of group 1 (1A, 1B and 1D) and 6 (6A, 6B and 6D) chromosomes [12]. Genes located in the three loci (Gli-A1, Gli-B1 and Gli-D1) of the group 1 chromosomes code for all ω-gliadins and most of γ-gliadins. While all α-gliadins, most of β-gliadins and some γ-gliadins are encoded by genes located in the three loci (Gli-A2, Gli-B2 and Gli-D2) of the group 6 chromosomes [12, 35]. Variation at each of these loci provides a large number of proteins.

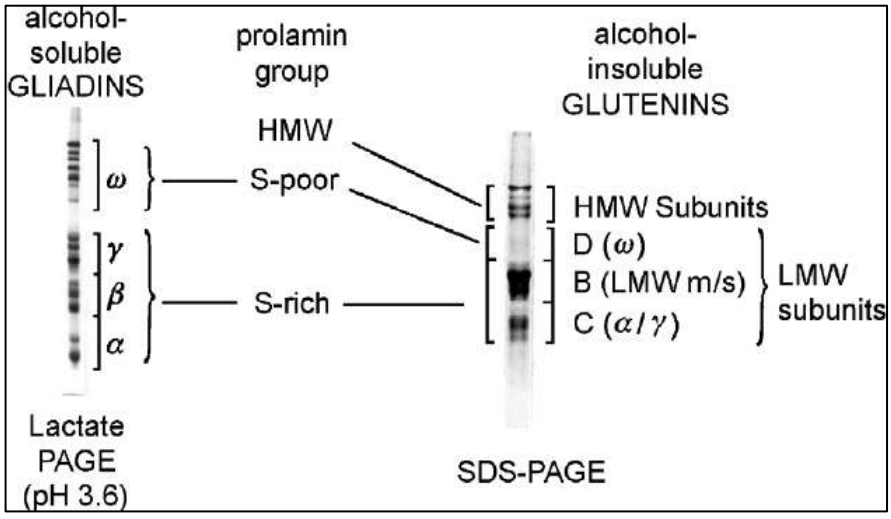

Figure 4. Classification of gluten proteins

1.5 Glutenin's

The glutenin fraction is formed by highly polymerized protein molecules linked together by interchain desulphated bonds. Native glutenin is HMW protein complex soluble only in dilute alkali or acid. However, after reduction of desulphated bonds, individual glutenin subunits are soluble in aqueous alcohols analogous to gliadins [10, 12, 28, 31]. MW of aggregated glutenin subunits is ranging from about 50,000 to more than 10 million (Figure 4). Glutenin complex is the major determinant of dough

properties and the amount of glutenin's in wheat flour correlates with dough strength. Glutenin's are classified into two distinct groups differing in MWs: LMW glutenin subunits (LMW-GS) and HMW glutenin subunits (HMW-GS). LMW-GS constitute predominant proteins within glutenin fraction. They represent about 20 % of total gluten proteins [28]. Despite of their abundance, they have been much less investigated than HMW-GS in the past. It was mainly due to overlapping between LMW-GS and gliadins in analysis by one-dimensional sodium dodecylsulphate polyacrylamide gel electrophoresis (1D-SDS PAGE). Nevertheless, modern analytical techniques and approaches involving PCR (polymerase chain reaction) analysis, mass spectrometry (MS) and proteomics, has enabled better research of LMW-GS [36-38]. With respect to MW and amino acid composition the LMW-GS are related to α/β- and γ-gliadins. LMW-GS also have two distinct N- and C-terminal domains. N-terminal domain consists of repetitive sequences rich in glutamine and proline, while C-terminal domain is homologous to C-terminal domain of α/β- and γ-gliadins [28]. LMW-GS contain eight cysteine residues, which are considered to be involved in the formation of both intra- and interchain disulphide bonds [28, 36]. For steric reasons, some cysteine residues are not able to form intrachain disulphide bonds and, consequently, interchain disulphide 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 interchain disulphide bonds [28, 39]. LMW-GS can be divided into B-, C- and D-type, based on their SDS-PAGE mobilities and isoelectric points (pI) [12, 37]. The B-type is the most abundant and middle-weight group within the LMW-GS. The C-type is faster and the D-type is slower compared to the B-type. 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 [12, 36]. The synthesis of some LMW-GS is also controlled by genes on the group 6 chromosomes [12]. HMW-GS are minor components within the gluten proteins family in terms of quantity but are major determinants of gluten elasticity. They account for about 10 % of gluten proteins [10, 28, 40]. HMW-GS are encoded at the Glu-A1, Glu-B1 and Glu-D1 loci located on the long arms of group 1 chromosomes (1A, 1B and 1D) [12]. All bread wheat cultivars have six HMW subunit genes, where each locus includes two genes linked together encoding two different types of HMW-GS: the x-type (1Ax, 1Bx and 1Dx) and the y-type (1Ay, 1By and 1Dy) subunit [10, 12, 28, 39-42]. However, some of these genes are not expressed, resulting in variations in HMW-GS number. HMW-GS vary in different wheat cultivars from three to five. It has been found that dough properties are strongly influenced by the quantity and the type of HMW-GS [10, 28, 40, 42]. The x-type subunits affect gluten elasticity more significantly than the y-type subunit. Generally, the x-type subunits have lower electrophoretic mobility in SDS-PAGE and higher MW (ranging from 83,000-88,000) than the y-type subunits (with MW from 67,000-74,000) [28, 40]. Structurally, HMW-GS consist of three

domains, small non-repetitive N- and C-terminal domains, and a large repetitive central domain [28, 39, 40]. Almost all cysteine residues are situated in the N- and C-terminal domains. Central domain comprises repetitive hexapeptides (consensus PGQGQQ), nonapeptides (consensus GYYPTSPQQ) and tripeptides (consensus GQQ). The hexapeptides and nonapeptides are present in both x- and y-type subunits, while the tripeptide motifs are present only in the x-type subunits [12, 40, 42]. Both LMW-GS and HMW-GS genes exhibit extensive allelic variations.

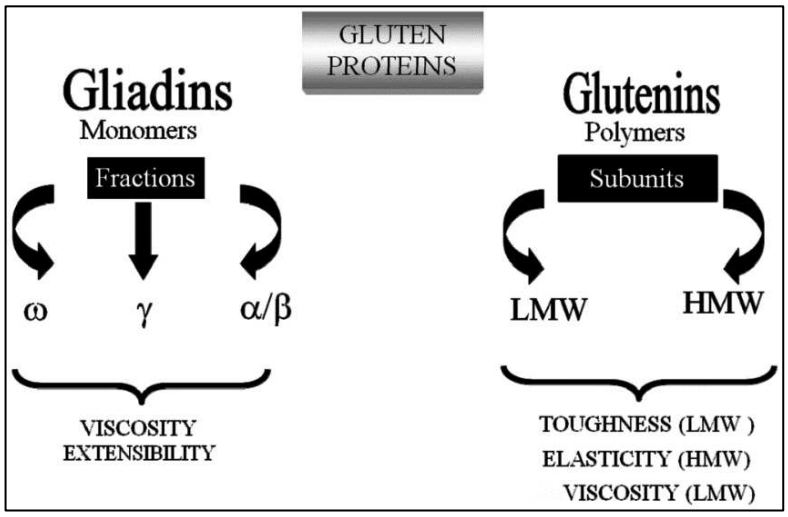

Figure 5. Gluten proteins: fractions and technological properties

From the biological point of view the role of gluten proteins is to provide a store of carbon, nitrogen and sulphur to support grain germination and seedling growth. Furthermore, these proteins determine bread making quality of the wheat (Figure 5). Finally, gluten proteins are also known as an environmental factor triggering celiac disease (CD) in genetically-predisposed individuals [43, 44].

2. Nutritional Quality of Wheat Flour

The therapy with gluten-free products, besides the risk of nutrient deficiency and metabolic syndrome, as described above, entails the difficulty of maintaining the cure over time. Reduced palatability and taste of gluten-free food create enormous limitations in the diet of patients. To solve these issues, numerous studies are currently devoted to the use of *in vitro* detoxified flour or flour from ancient wheat cultivars, in the formulation of pasta and baked goods. Worldwide, the number of people who eat wheat for a substantial part of their diet reaches several billions. 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. Despite its relatively low protein content (usually 8%–15%), wheat still provides as much protein for human and livestock nutrition as the total soybean crop (as calculated in reference [45]. However, the lysine content of wheat is low and varies

significantly from grain to flour [46]. Grain of high protein content has very low content of lysine approximately 30 mg g^{-1} protein [47]. Wheat is a source of minerals such as Zn (20–30 mg Kg^{-1}) and Fe (30–36 mg Kg^{-1}), contributing to 44% of the daily intake of iron (15% in bread), and 25% of the daily intake of zinc (11% in bread) in the UK [48] Wheat is also a source of selenium which varies widely from about 10 µg Kg^{-1} to over 2000 µg Kg^{-1}(FAO/WHO, 2001; [49]). The concentration of selenium in wheat is largely determined by the availability of this element in the soil. Wheat produced in Western Europe may contain only one-tenth of the selenium that is present in wheat grown in North America. Wheat also contains a range of components with established health benefits that are concentrated, or solely located, in the bran. In addition, the following components are either present in low amounts, or completely absent, and with a large variation in their concentrations.

2.1 Detoxification of Wheat Gluten

Numerous studies 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). A 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 [50]. Such an approach, carrying out a 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 starch, emulsifiers or hydrocolloids.

Another method to detoxify gluten proteins uses the specific transdamidation of toxic epitopes done by the tissue-transglutaminase of microbial origin (*Streptomyces mobaraensis)* in the presence of lysine methyl ester [51]. 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 [52]. 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.

3. Celiac Disease

3.1 Overview of Celiac Disease

Celiac disease is a chronic intestinal disorder induced in sensitive individuals by protein complex called gluten, which is found in wheat and, to a lesser extent in barley, rye and possibly oats [53-54]. This condition is known under several names: coeliac disease, coeliac sprue, non-tropical sprue, endemic sprue, gluten enteropathy or gluten-sensitive enteropathy, gluten intolerance and idiopathic steatorrhea. CD is also defined as autoimmune mediated enteropathy of small intestine in genetically-predisposed individuals. Ingestion of food containing gluten proteins causes inappropriate immune response leading to small intestine inflammation with typical destruction of villous structure (Figure 6), which results to malabsorption of nutrients [44]. As the almost all nutrients (amino acids, carbohydrates, minerals, vitamins, etc.) are absorbed in the small intestine, the damage of the gut villi leads to a wide range of symptoms in CD: weight loss, chronic diarrhea, bone pain, fatigue, anemia, delayed growth, etc. The symptoms can differ person to person, which complicates the diagnosis of CD. Moreover, many symptoms of CD are similar to those of other diseases, e.g. intestinal infections, Crohn`s disease, chronic fatigue syndrome, etc. [54, 55-65].

It is generally accepted that CD is the most common chronic inflammatory intestinal disease, which confirm recent studies [57-63]. CD has been recognized as a world-wide health problem because many foods are based on cereals that contain proteins toxic to patients with CD. The prevalence of all forms of CD is 1:100-300 in general population [53, 54, 59, 61-63]. Until now the only effective treatment of CD is based on strict elimination of mentioned cereals from diet of celiac patients for a lifetime.

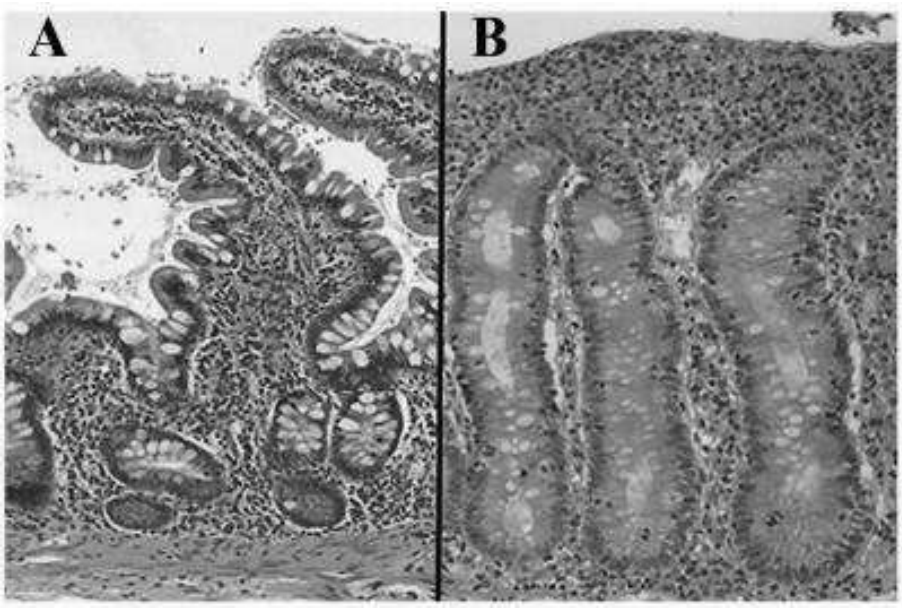

Fig. 6. Small intestine biopsy: A - normal small intestine, B - total villous atrophy of CD patient.

3.2 Historical background

The first reference about CD dates back to the second century A.D., when Greek physician Aretaeus of Cappadocia reported the first acceptable description of CD [44, 62, 72, 73]. The name "sprue" was firstly used in 18th century and is derived from Dutch word spruw, meaning aphthous disease, due to high prevalence of aphthous mouth ulcers in celiac patients. Findings of Aretaeus of Cappadocia were translated from Greek to English for the Sydenham Society of England in 1856 by Francis Adams [72, 73]. Adams introduced the term "coeliac", recently more often spelled as celiac. In 1888 Dr. Samuel Gee, a paediatrician, presented the scientific paper called "On the Coeliac Affection", which was the first modern-day description of CD [61, 63, 72-74]. He also mentioned the importance of diet control in CD. However, he did not mention anything about avoiding of cereals from human diet. Dutch paediatrician, Willem Dicke, observed that condition of individuals with CD improved significantly during the cereal shortage of World War II. When the cereal supplies were restored, the patients with CD relapsed. He noted that removal of wheat from diet of celiac patients resulted in complete disappearance of all symptoms of CD [61-62, 65, 56, 75- 73]. He reported his findings in the thesis in 1950 [69]. In 1954 British physician Paulley described the intestinal lesion (villous atrophy) in patients with CD [44-54]. The development of small intestine biopsy in 1950`s, confirmed the small intestine as target organ in CD [62, 68, 70]. The discovery of genetic, immune and molecular mechanisms in CD has distinctively increased the knowledge about this disorder in the last 25 years.

3.3 Immunopathogenesis

Celiac disease is multifactorial disorder with genetic predisposition. The disease is strongly associated with human leukocyte antigen (HLA) genes. HLA genes, the most polymorphic human genes, are located within the gene cluster called major histocompatibility complex (MHC) on chromosome 6 [55, 56, 71, 72]. The MHC region covers over 200 genes and many of them are related to the function of immune system. In humans, this region is divided into three classes: MHC I, MHC II and MHC III. While the MHC class I molecules are expressed by almost all nucleated cells of the human body, the expression of the MHC class II molecules is restricted to professional antigen-presenting cells (APCs) of the immune system, such as B cells, macrophages and dendritic cells. MHC class III region covers a diverse group of genes, many of which are involved in the immune system. The MHC class I and II molecules act as recognition molecules and antigens in the immune system. These molecules bind peptide fragments derived from pathogens and present them to antigen-specific cells. CD is primarily associated with the MHC class II in the loci HLA-DQ. The HLA-DQ alleles encode specific HLA-DQ2 or HLA-DQ8 molecules (heterodimers) that bind the gluten derived peptides and present them to antigen-specific T cells [65]. The HLA-DQ2 heterodimer is formed by α chain encoded by the allele HLA-DQA1*05 and β chain encoded by the allele HLA-DQB1*02. Up to 95% individuals

with CD carry HLA-DQ2 heterodimer encoded either in cis (in DR3 homozygous individuals) or in trans (in DR5/DR7 heterozygous individuals) configuration [44, 55, 72, 74-79]. Majority of remaining patients with CD carry the HLA-DQ8 heterodimer formed by α chain encoded by the allele HLADQA1*03 and β chain encoded by the allele HLA-DQB1*0302 [44, 72, 77]. Although, the frequency of HLA-DQ2 in European population is high (20-30%), only minority of these individuals (approximately 40%) will develop CD. This means that HLA-DQ is necessary but not sufficient for development of CD. Due to this fact, the HLA testing is not useful as screening test for CD [44, 55, 72]. On the other hand genetic testing for HLA-DQ can be used as an effective test excluding CD in any individual. The next investigations have been focused to identify additional non-HLA genes associated with CD, and more recently, the linkage of CD with some others chromosomes was found [58, 72, 76-80]. It is well known that ingestion of gluten proteins in genetically susceptible individuals results to inappropriate T cell mediated immune response taking place in two compartments, the lamina propria (CD4+ T cells) and the epithelium (CD8+ T cells) [58]. T lymphocytes (cells) together with B lymphocytes (cells), the special types of leukocytes, are central cellular elements of adaptive immunity. B cells are involved in the humoral immune response, whereas T cells are involved in cell-mediated immune response [81, 82]. All mature lymphocytes (both B and T cells) carry receptor molecules on their surface that recognize specific target (Fig. 6). B lymphocytes carry B cell receptors (BCR) and T lymphocytes carry T cell receptors (TCR). TCR is a disulfide heterodimer consists of one α− and one β−chain. The other form has one γ− and one δ− chain [75, 82]. T cells are able to recognize pathogen only when the antigens (small fragments of the pathogen) were processed and presented in combination with a receptor called MHC molecules (also known as HLA molecules). On the other hand, BCR on the B cell surface is, in fact, an antibody (immunoglobulin) recognizing whole pathogen without antigen processing. If each lineage of B cells expresses different antibodies then the complete set of BCRs represents all the antibodies that the human body can produce [81, 82]. There are two major subtypes of T cells: cytotoxic (killer) T cells (Tc) and helper T cells (Th), carrying either CD8 or CD4 co-receptor molecules on their surface, respectively (Fig. 6). Tc cells (CD8+ T lymphocytes) only recognize antigens bound to class I MHC molecules, while Th cells (CD4+ T lymphocytes) only recognize antigens bound to class II MHC molecules [81]. The function of minor subtype of T cells, γδ T cells, has not been clarified. Furthermore, Th cells are divided functionally into Th1 and Th2 by the pattern of cytokines (bioactive polypeptides influencing behaviour of target cell) that these cells produce. Th1 cells are characterized by production of interferon-γ (a cytokine), which is linked to mucosal damage in CD patients [58, 72, 75-86]. Cytokines associated with Th2 cells are involved in activation of B cells to produce antibodies. CD4+ T lymphocytes that can be isolated from the intestinal mucosa of untreated patients suffering from

CD are not found in the small intestine of healthy individual or in patients with CD on a gluten-free diet (GFD). This implies that CD4+ T lymphocytes play a central role in the pathogenesis of CD, although the actual mechanisms responsible for mucosal damage are not understood well yet [58]. The HLA class II molecules DQ2 and DQ8, which are found on APCs, bind gluten derived peptides with variable length (epitopes) and present them to CD4+ T lymphocytes in the lamina propria of the small intestine (Fig. 6). When the interactions between gluten derived peptides, T cells and HLA-DQ molecules were determined, it was found that HLADQ2 and -DQ8 molecules have preference for peptides with negatively charged residues. Seeing that the gluten proteins contain only few amino acids with negative charge, it was secret for a long time by which means the HLA-DQ2 and -DQ8 molecules bind peptides generated from gluten proteins in the intestinal tract. This question was solved after discovery of tissue transglutaminase (tTG) as the major target autoantigen of autoantibodies present in the sera of patients with untreated CD [85-88]. tTG is a calcium-dependent enzyme present in most cells of human body, which is released during cellular damage (e.g., small intestine inflammation in CD). It cross-links glutamine residues in one protein with lysine residues of other protein in order to control the tissue damage. In the absence of an appropriate substrate (protein with lysine residues), the tTG activity leads to conversion of glutamine residue to glutamic acid. Moreover, it was found that tTG deamidates only selected glutamine residues of the gluten-derived peptides. The spacing between proline and targeted glutamine residues seems to play a key role in the tTG deamidation specificity. But only certain deamidated gluten-derived peptides have increased affinity to HLA-DQ2 and -DQ8 molecules [88-94]. However, deamidation is not necessary requirement for T cell stimulation in CD [58, 72]. Gluten proteins are known as an important environmental factor triggering CD in genetically-predisposed individuals. These proteins have several unique features. They are characterized by extremely high content of the amino acids proline and glutamine. High proline content makes them exceptionally resistant to proteolytic digestion by gastric, pancreatic and intestinal proteases within the gastrointestinal tract, because those enzymes have no prolyl endopeptidase activity. This results to relatively large gluten-derived peptides with a high content of proline and, in particular, glutamine amino acids. Such peptides represent suitable substrates for tTG [72, 86, 94]. As was mentioned above gluten constitutes a highly complex mixture of heterogeneous proteins divided into two groups depending upon their solubility – prolamins and glutelins. Several studies confirm that both these groups of proteins contain sequences responsible for stimulation of celiac mucosal T cells (epitopes) [60, 85, 95-97]. Development of sophisticated analytical techniques, including MS, in last fifteen years has brought considerable progress in identifying of amino acid sequences of the gluten peptides (epitopes) that may trigger CD.

Majority of recent studies have been focused on determination of toxic peptides (in terms of CD) of wheat storage proteins, especially gliadins. There is a large number of T cell stimulatory epitopes in wheat gluten proteins (approximately 50) that were found on the basis of in vivo and in vitro testing of natural, synthetic and recombinant peptides from wheat [99-106]. As result from recent studies, all toxic peptides have (besides the glutamine) high content of the proline, which was found to be an essential for immunogenic properties of gluten peptides [95]. Although some studies described certain tetrapeptide sequences (like PSQQ and PQQQ) essential for toxicity, other studies reported immunogenic peptides, which do not contain any of these sequences. In 2002 Shan et al. described the 33-mer peptide (within the 266 aminoacid long sequence of α2-gliadin) as an immunodominant gluten peptide, which is resistant to degradation by gastric, pancreatic and intestinal proteases. After deamidation by tTG, the 33- mer peptide is extremely stimulatory all gliadin-specific T cells [105-107]. This peptide (sequence LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF, underlined Q residues correspond to tTG deamidation sites) contains several short sequences, in part overlapping, previously described as the T cell stimulatory epitopes (Figure 7).

These epitopes have lower T cell stimulatory activity than the 33-mer peptide, which corresponds with finding that large gluten peptides containing several HLA-DQ binding sequences have greater T cell stimulatory activity than small peptides containing single HLA-DQ binding sequence [72, 107]. Finally, gluten proteins contain many peptides with T cell stimulatory capability. While some of gluten-derived peptides are capable to elicit T cell responses in almost all CD patients (those peptides are immunodominant), others appear to be less immunogenic.

Generally, CD is caused by dietary of wheat gluten proteins (gliadins and glutenins) and related prolamin proteins of barley (hordeins) and rye (secalins) in susceptible individuals. Although barley and rye have not been studied as extensively as wheat, few studies have been done on the toxicity of barley and rye proteins for patients with CD [92, 106]. Oat and its toxicity for CD patients is widely discussed question in recent years. Oat prolamins (avenins) are considered to be less harmful than related proteins of wheat, barley and rye. Moreover, oat is taxonomically more distantly related to wheat, barley and rye (Fig. 7). Traditionally, oat has been excluded from the GFD. Nevertheless, there are some studies indicated that oat is harmless for CD patients [107]. Other studies show that the moderate amounts of oat in GFD is harmless for majority of CD patients [67]. On the other hand, even if the oat is well tolerated by many CD patients, there are still patients intolerant to ingestion of oat. In addition, the commercial oat products are often contaminated by wheat, barley and rye. For this reason the oat should be excluded from the diet of CD patients [108, 109]. Finally, 30 other members of grass family (e.g., rice, maize, sorghum and millet) are considered to be safe in relation to CD.

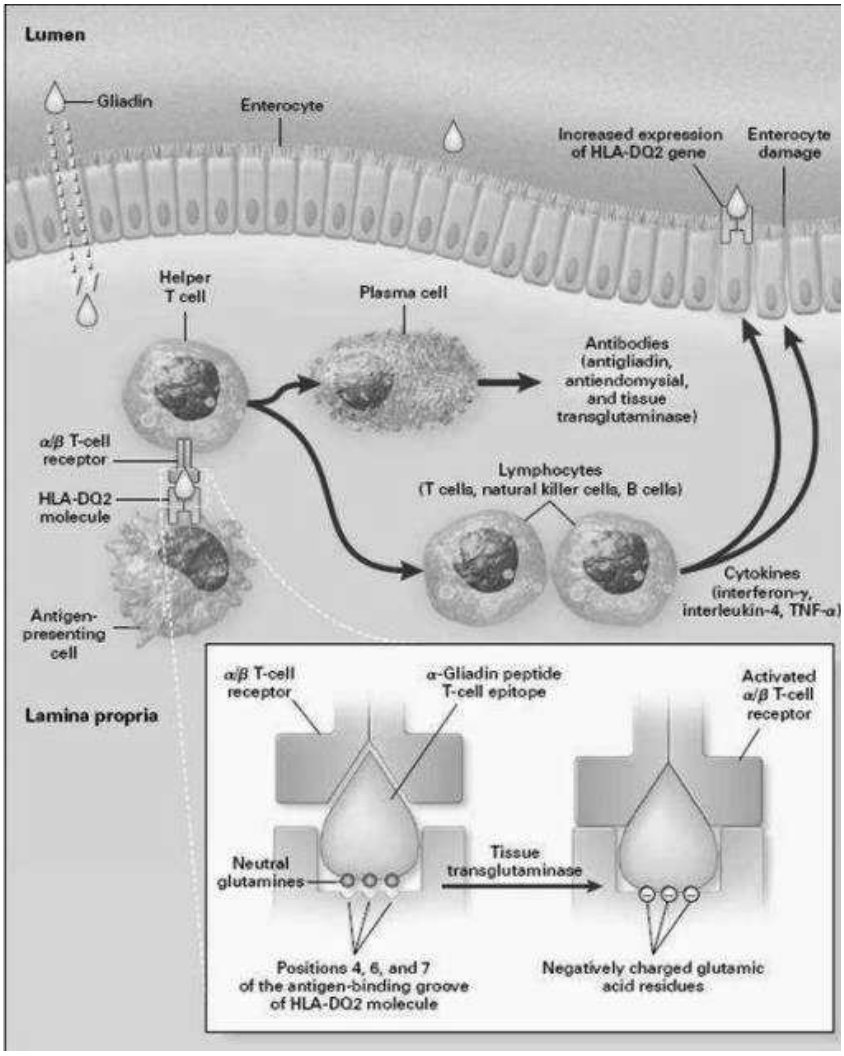

Figure 7. Pathogenesis of Celiac Sprue (Farrell and Kelly, 2002).

3.4 Clinical manifestations

Clinical manifestations of untreated CD are highly variable, involving multiple organ systems, and may present at any age. Nevertheless, they present most commonly in early childhood (usually in infants less than two years of age) after introduction of cereals to the diet. The second peak age of diagnosis is around forty years [61, 63]. Typical gastrointestinal manifestations include diarrhea, weight loss, failure to grow, vomiting, abdominal pain, bloating and distension, anorexia and constipation. Gastrointestinal symptoms may be, however, absent or less pronounced. Extraintestinal (non-gastrointestinal) manifestations such as iron deficiency anemia, fatigue and malaise, dermatitis herpetiformis, osteoporosis, infertility, vitamin deficiencies and neurological symptoms, are most present in adulthood [58, 59, 109]. Iron deficiency anemia has been found the most common clinical manifestation in adults with CD. CD is usually divided into four clinical subtypes [109]: Classical CD represents gastrointestinal malabsorption symptoms. The diagnosis is confirmed by both

serological testing and biopsy. Symptoms improve on GFD. Classical CD is a common presentation in the childhood. CD with atypical symptoms is characterized by predominance of extraintestinal manifestations with few or no gastrointestinal symptoms. At the present time it is the most common presentation of CD. The diagnosis is established by serologic testing and biopsy. As with classical CD, symptoms improve on GFD. Silent CD refers to individuals who are apparently asymptomatic, but have positive serological test and villous atrophy revealed by biopsy. These individuals are usually detected via screening of high risk groups and villous atrophy may be detected, eventually, by endoscopy and biopsy performed for another reason. Latent CD is defined by positive serological test, but negative biopsy results. These individuals are asymptomatic at the time of diagnosis, but later may develop symptoms and/or histological changes. Untreated CD is associated with a number of diseases and complications [110]. Recent screening studies have shown that autoimmune diseases occur more frequently in patients with CD in comparison to general population. For example, the prevalence of CD in patients with type 1 diabetes mellitus is up to 8 % [111]. Another example of autoimmune condition associated with CD is thyroiditis. Other associated diseases are IgA deficiency, hepatitis, rheumatoid arthritis, Down`s syndrome, Crohn`s disease, epilepsy, and many others [111]. In addition, patients with CD are at increased risk of malignancy and mortality [112-114]. Specific manifestation of CD is refractory sprue. This state is defined as a symptomatic severe villous atrophy of the small intestine with no improvement after a strict GFD [115]. Refractory sprue can be associated with a number of complications such as enteropathy-associated T cell lymphoma, ulcerative jejunoileitis, and collagenous sprue. Patients with refractory sprue typically undergo treatment with corticosteroids and immunosuppressants. The ultimate treatment, in the case of patients who do not respond to treatment with corticosteroids and immunosuppressants, is total parenteral nutrition. 4.2.5 Diagnosis CD was originally considered to be relatively uncommon disorder. However, recent screening studies based on serologic tests have revealed that prevalence of CD approaching 1 % of the population [45, 51, 53, 55, 104]. The “celiac iceberg” is a common model illustrating the epidemiology of CD. The tip of iceberg constitutes individuals with clinically recognized disease. Greater proportion of the iceberg corresponds to patients with silent and latent CD, and to individuals having the heritage but with normal intestinal histology [105, 108]. Definitive diagnosis of CD is based on 3 key parameters: (1) positive serologic test, (2) characteristic findings on the small intestine biopsy and (3) clinical and serological improvement after the GFD [47, 49, 55, 63]. Small intestine biopsy is the gold standard in diagnosis of CD. The histological findings on the biopsy include varying degrees of villous atrophy, crypt hyperplasia, and increased intraepithelial lymphocytes. As the pathological interpretation of intestinal biopsies is a major pitfall in the diagnosis of CD, the multiple biopsies need to be obtained to confirm histological findings. Histological

changes of small intestine are not specific for CD and may respond to other conditions such as tropical sprue, giardiasis, or acute viral gastroenteritis. Therefore, the biopsy alone is not sufficient for diagnosis of CD.

Currently available serologic tests include immunoglobulin A endomysial antibody (IgAEMA), IgA tissue transglutaminase antibody (IgA-tTG), IgA and IgG antigliadin antibodies (IgA-AGA and IgG-AGA). EMA is detected using an immunofluorescent assay, which is qualitative assay, so results are either positive or negative. tTG is detected using an enzyme-linked immunosorbent assay (ELISA). Although the results of EMA and tTG tests can vary depending on laboratory and severity of CD, both these tests are highly sensitive and specific [110, 111]. In CD patients with IgA deficiency the IgG-EMA and IgG-tTG tests should be performed. Antigliadin antibody (IgA-AGA and IgG-AGA) tests are less sensitive and specific compared to IgA-EMA and IgA-tTG antibodies. Therefore, the use of antigliadin antibodies as a screening test is no longer recommended, except in special clinical circumstances. Currently, the only effective treatment of CD is life-long adherence to strict GFD. Elimination of wheat, barley, rye and oats from the diet usually results in symptomatic and histological improvement. Most patients show rapid improvement of symptoms within weeks, while histological improvement can take several months to years. Response to GFD depends on a number of factors and, therefore, varies from one patient to another [44, 50]. However, some patients do not respond to GFD. Those patients should undergo systematic evaluation. The most common reason is an incomplete adherence to GFD. It should be noted that the complete elimination of gluten is not easy task. Gluten is present in many commercial and processed foods, which is not always declared properly. For example, wheat flour is widely used as a thickener in many commercial products. In patients who still have the symptoms despite the strict GFD is very important to rule out alternative disorder such as irritable bowel syndrome, chronic infections, pancreatic insufficiency, etc. Another reason for why some patients do not respond to GFD is refractory CD [115]. In addition to gluten restriction, the newly diagnosed patients having clinically evident malabsorption should be initially treated with appropriate supplements (e.g., multivitamin, iron, calcium, vitamin D) to correct deficiency states [62]. As the strict GFD is very difficult to achieve and maintain, some other approaches are being studied in order to reduce the necessity of GFD. One of the most promising areas of current research is peptidases, especially microbial prolyl endopeptidases [119-122]. Prolyl endopeptidases represent a unique class of serine proteases with the ability to cleave peptides at internal proline residues. These enzymes can degrade gluten to non-toxic components. Therefore, prolyl endopeptidases have a great potential to treat CD by reducing or eliminating the immunostimulatory peptides from the small intestine. Another alternative to GFD are breeding programs (and possibly genetic engineering) aimed at generating cereal varieties acceptable for

individuals suffering from CD [123]. This, however, can interfere with the fact that gluten proteins play a key role in dough properties. Recently was described decapeptide from durum wheat (sequence QQPQDAVQPF) able to inhibit the abnormal immune response triggered by gliadin peptides in CD [123]. This finding suggests an alternative therapeutic strategy for CD based on peptides naturally occurring in toxic cereals.

4. Gut microbiota in gut homeostasis

At birth, we are colonized with a complex community of microbes that reaches up to a density of 1×10^{12} bacterial cells per gramme of content in the adult colon (Figure 8). These microbes live in a symbiotic relationship with the host and are key determinants of health and disease by influencing nutrient absorption, barrier function and immune development. Even though the bacterial load in the colon is markedly higher than in the small bowel, evidence exists that the microbiota of the small bowel is in closer contact with the host because of a loose mucus layer, and that it has a critical role in shaping the immune system and inducing the production of antimicrobial peptides that in turn affect the colonic microbiota [124]. A comprehensive study of the gut microbiota using culture-independent approaches determined that, unlike previously thought, the small intestine harbours facultative and strict anaerobes [125]. Although less complex than the microbiota of the colon, *Clostridium* spp., *Streptococcus* spp. and coliforms are dominant groups in the small intestine. Moreover, this study indicated that the small intestinal microbial community rapidly responds metabolically to dietary changes [125].

Studies using germ-free mice have demonstrated the importance of the microbiota on the development of host physiology and a functional immune system (Figure 9). In addition to gut morphological and functional differences, germ-free animals have immature organized lymphoid tissues [126-128], as well as reduced numbers of intestinal $CD4^+$ T cells, small intestinal type 17 T helper (T_H17) cells [129], colonic regulatory T (T_{REG}) cells [130]and T-cell receptor (TCR)$\alpha\beta^+$ intraepithelial lymphocytes (IELs) [131,132] compared with colonized mice. Differentiation of B cells to IgA-producing plasma cells is also dependent on the microbiota [133,134]. Differentiation of B cells to IgA-producing plasma cells is also dependent on the microbiota. [133,134] Signals from the microbiota induce production of antimicrobial peptides (AMPs), such as RegIIIγ, from Paneth cells, $\gamma\delta TCR^+$ IELs and epithelial cells [135-137]. The gut microbiota also stimulates the release of mucins from goblet cells [126] The microbiota might also be critical for the development of various innate lymphoid cell (ILC) subsets, and for the production of IL-22 from group 3 ILCs [138], which in turn stimulates AMP release from epithelial cells [139,140]. Thus, microbiota–host interactions are key in the development of normal functional and immune responses to gut luminal antigens.

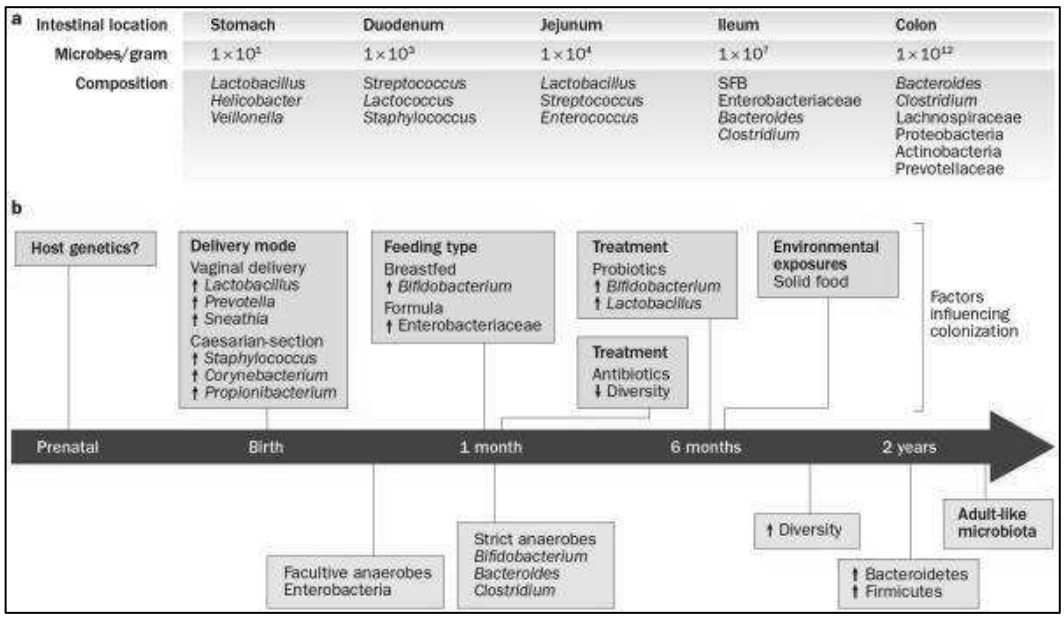

Figure 8. Development of the gut microbiota. The composition and density of the microbiota varies along the length of the intestine as well as with age. **a** | Differences in microbial composition and density are observed along the length of the gastrointestinal tract, with much lower densities and greater variability in the proximal intestine. **b** | The gut microbiota fluctuates over the first 2–3 years of life, with high interindividual variability and low diversity, but becomes more stable over time.

When studied individually, particular members of the gut microbiota can differentially modulate host responses (Figure 9). For example, flagellin, a bacterial structure that stimulates Toll-like receptor (TLR)5, can stimulate RegIIIγ production from epithelial cells via IL-22 release from dendritic cells [141]. Flagella are associated with pathogenicity by promoting bacterial motility, cell adhesion and biofilm formation, constitute a virulence factor that can modulate host immune responses and are present in bacteria such as *Escherichia coli* and *Salmonella* [142,143].

Furthermore, segmented filamentous bacteria (SFB) are potent inducers of T_H17 cells in mice [140,141]. A murine community of eight commensals, or altered Schaedler flora, induce balanced immune responses, which includes T_{REG} cells as well as T_H17 cells, but to a lesser extent than SFB. On the other hand, monocolonization of mice with *Clostridium* or *Bacteroides fragilis* induces colonic T_{REG}-cell differentiation [146-148]. Bacterial products, such as *B. fragilis*-derived polysaccharide A or short-chain fatty acids (SCFAs; for example, acetic acid, propionic acid and butyric acid), have also been shown to induce T_{REG} cells [152].

Products of bacterial metabolism (SCFAs) have also been shown to induce IL-18 production from epithelial cells and promote tolerogenic dendritic cells, which produce IL-10 and retinoic acid [153].

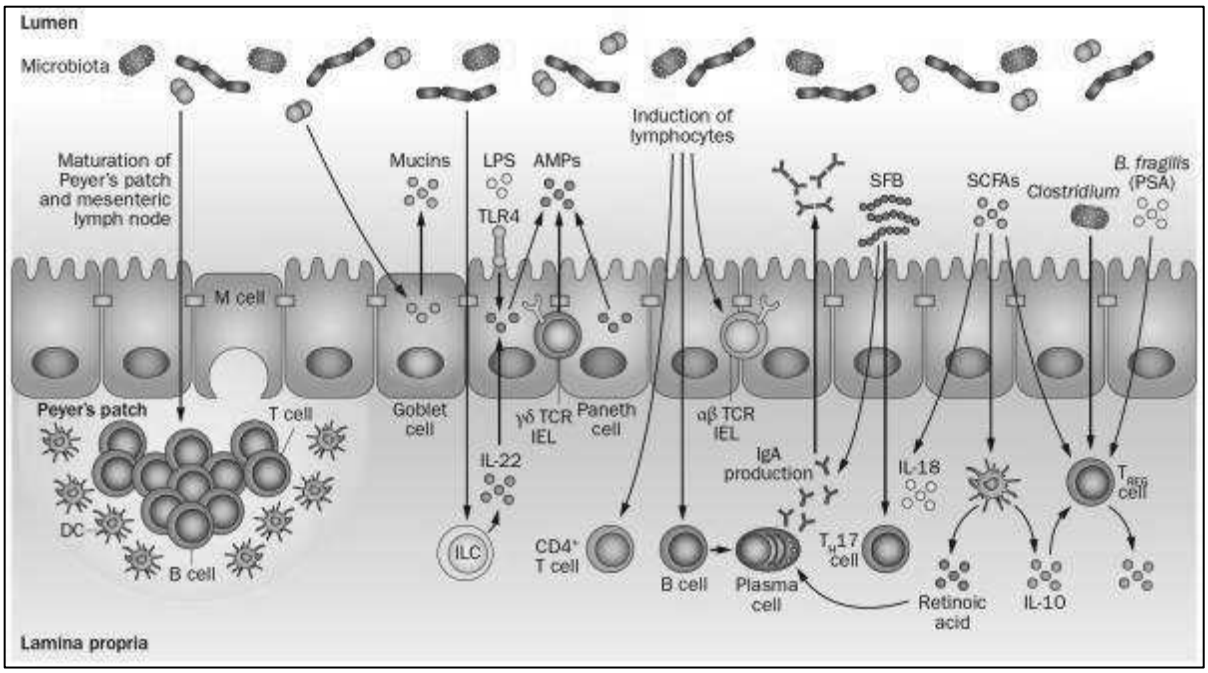

Figure 9. Gut microbiota shapes host immunity. The gut microbiota induces maturation of the gastrointestinal lymphoid tissue (Peyer's patches, MLN). Signals from the microbiota induce production of AMPs, such as RegIIIγ, from Paneth cells, γδTCR$^+$ IELs and epithelial cells. Microbial signals can also stimulate the development of ILC subsets, including IL-22-producing ILCs. Flagellin or LPS can stimulate AMP production from epithelial cells via IL-22 or TLR4, respectively. The gut microbiota also stimulates the release of mucins from goblet cells, and microbes influence the development of T-cell subsets, including CD4$^+$ T cells, αβTCR$^+$ IELs, and are critical for the induction of IgA-producing plasma cells. and SCFAs stimulate T_{REG} -cell differentiation. SCFAs can also promote IL-18 production from IL-18 production from epithelial cells and promote IL-10 and retinoic acid production from DCs, which in turn promotes differentiation of T_{REG} cells and IgA-producing plasma cells.

Overall, these studies suggest that induction of immune responses 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. Thus, given the importance of host–microbial interactions on host immunity and physiology, disruptions in gut microbiota composition or function (dysbiosis) might have important implications for health and disease. Indeed, dysbiosis has been described in a number of chronic inflammatory diseases [153-154]. However, the overall contribution of dysbiosis from disruption of homeostasis to disease development is not well understood.

4.1 The gut microbiota in coeliac disease

Approximately 30% of the general population carry the HLA-DQ2/8 coeliac disease susceptibility genes; however, only 2–5% of these individuals will go on to develop coeliac disease, suggesting that additional environmental factors contribute to disease development [155]. The additional factors that influence coeliac disease development are unknown, but might include alterations in the intestinal

microbiota. Indeed, some studies have demonstrated that patients with active coeliac disease have altered faecal and duodenal microbiota compositions compared with healthy individuals, which is partially restored after treatment with a gluten-free diet. Specifically, changes in the abundance of Firmicutes and Proteobacteria have been detected in children and adults with active coeliac disease [156-157]. Other studies have reported decreases in the proportion of protective, anti-inflammatory bacteria such as *Bifidobacterium*, and increases in the proportion of Gram-negative bacteria such as *Bacteroides* and *E. coli*, in patients with active coeliac disease [160]. Increases in the number of *Staphylococcus* and *Clostridium* [161] and decreases in *Lactobacillus* spp. have also been reported in children with coeliac disease. Altered diversity and altered metabolic function (SCFAs) of the microbiota have also been reported in patients with coeliac disease [164]. A study demonstrated that the microbial composition of the gut in patients with coeliac disease was associated with the clinical manifestation of disease. The gut microbiota in patients experiencing gastrointestinal symptoms was dominated by Proteobacteria, whereas the microbiota of patients with dermatitis herpetiformis or individuals experiencing dyspepsia (controls) was dominated by Firmicutes [157]. Increases in the number of Proteobacteria were also detected in patients with coeliac disease who were experiencing persistent symptoms, despite having normal histology and adhering to a gluten-free diet [165]. Together, these studies demonstrate that there are differences in microbial composition between patients with coeliac disease and healthy individuals as controls; however, the literature has not revealed a typical `coeliac microbiota signature'. This scenario is not unlike other chronic inflammatory gastrointestinal diseases, such as IBD or IBS, for which evidence supports an association between altered microbial composition and disease states [165]. However, consensus across studies with respect to the specific changes involved is lacking and a disease-specific microbial signature has not yet been defined [166-168] 8figure 10). Differences in the age of the study population (children versus adults), methodology (fluorescence *in situ* hybridization-PCR, denaturing gradient gel electrophoresis, 16s ribosomal RNA sequencing), sampling technique (biopsy versus faecal sample), length of gluten-free diet and the clinical presentation of disease could contribute to inconsistent findings in the literature. These differences make it difficult to compare across studies and determine whether the gut microbiota contributes to coeliac disease development or progression, or whether it is simply a consequence of the disease. Moreover, the exact mechanisms through which the gut microbiota might influence coeliac disease onset or progression is unknown, but could include activation of innate immune system, modulation of the epithelial barrier, or exacerbation of the gliadin-specific immune response.

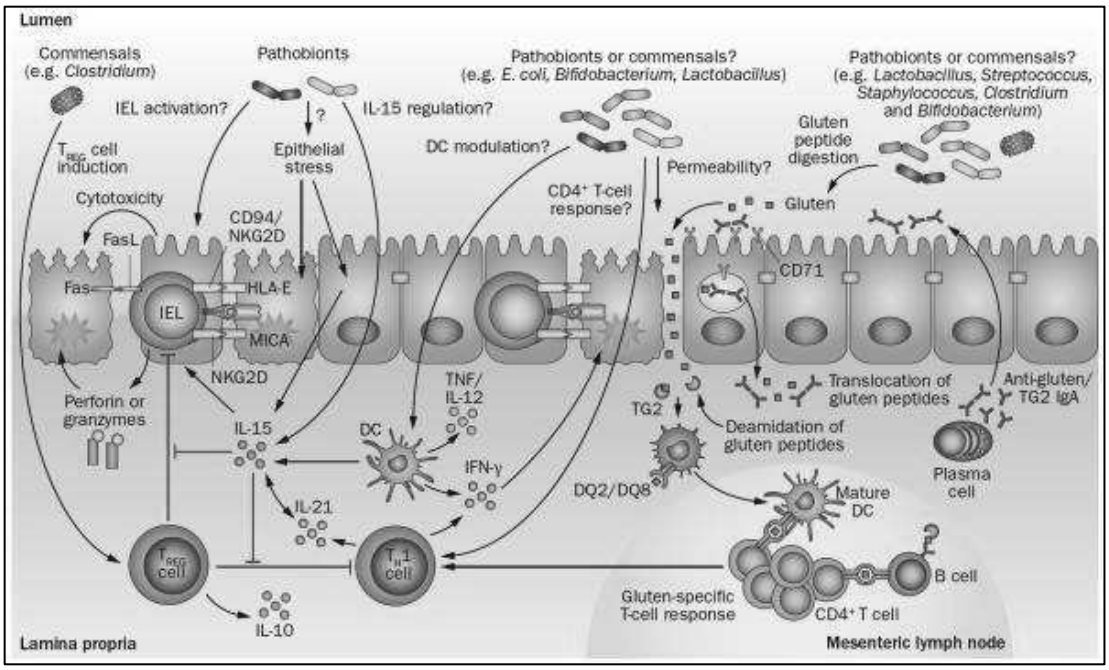

Figure 10. Potential microbial modulation of coeliac disease pathogenesis. Gluten peptides in the small intestinal lumen translocate the epithelial barrier, via transcellular or paracellular mechanisms and are deamidated by tissue TG2 in the lamina propria. Deamidated gliadin peptides are taken up by lamina propria DCs, inducing a proinflammatory gluten-specific $CD4^+$ T-cell response, characterized by IFN-γ and IL-21 production, and anti-gliadin and anti-TG2 antibody production by B cells in genetically predisposed hosts. Activation of the innate immune response is also a key initial step in coeliac disease.

5. "Gluten Friendly™" Technology

"Gluten Friendly™" technology is a method for the detoxification of gluten proteins from grains of cereals, in particular from the grains of wheat, aimed to obtain detoxified flours for the preparation of bread and pasta products, from wheat, preferably suitable for the alimentation of patients with celiac disease, but also adequate for its organoleptic characteristics and for its aspect, for the alimentation of the whole population. 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". This technology implies 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 so to induce changes in proteins that reduce the immunogenicity in vitro of the most common epitope involved in cealic disease (Lamacchia et al., 2015a Italian Patented Method N. 0001414717 (2015)[169]. Metodo per la detossificazione delle proteine del glutine dalle granaglie dei cereali. Inventors: Lamacchia, C., Di Luccia, A., Gianfrani, C (Lamacchia et al., 2015b; Lamacchia, C., Di Luccia, A. & Gianfrani C. (2015). Method for the detoxification of gluten proteins from grains of cereals. BioVaria Congress,

Europe's top technologies, Munchen, Germany, https://www.biovaria.org/past-event/ technologies/)[170] preserving the technological properties of the flour.

The "Gluten Friendly™" technology is based on the analysis of recent studies in which Lamacchia and others (2010) have reported that, when the high temperatures are applied to the caryopsis of wheat, the proteins undergo changes that are not similar to those seen in model systems, consisting only of gluten (Schofield et al., 1983; Singh and MacRitchie, 2004), nor to those seen in the pasta during the drying cycles. In particular, albumins and globulins are not incorporated in the polymers of high molecular weight but coagulate and interact with gliadins forming an aggregate of molecular weight intermediate to that of gliadins and albumins and globulins revealed as a new peak called "Intermediate Protein" (IP) peak. The participation of omega-gliadins to these changes suggests that the interaction between the proteins takes place not only through the formation of disulfide bonds but also through the formation of covalent bonds involving tyrosine residues.

The researchers Lamacchia and others (2010) explained this phenomenon on the basis of the fact that in the caryopsis of wheat, gluten is not yet formed and gluten proteins are deposited in different protein bodies (Rubin et al., 1992, Krishnan et al., 1986; Lending et al., 1989). In a recent study, Tosi and others (2009) confirmed, in fact, that the HMW are particularly abundant in the innermost layer of the caryopsis of wheat (endosperm) and practically absent in the subaleuronic layer which, however, is rich in gliadins and LMW. This pattern of deposition is maintained throughout all the development phase of the caryopsis of wheat and continues even after the merger of protein bodies and the formation of the starchy matrix.

Therefore, the segregation of gluten proteins in protein bodies when they are in the caryopsis and the application of high temperatures in this stage before the milling, would allow such proteins of experiencing structural changes such as not to make them recognizable anymore by intestinal transglutaminase, thereby blocking the waterfall of inflammatory cytokine. The technology has since been 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.).

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Chapter 2

Changes in wheat kernel proteins induced by microwave treatment

Changes in wheat kernel proteins induced by microwave treatment

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Abstract

Wheat kernels were subjected to microwave treatment, and the proteins were characterized by size exclusion high-performance liquid chromatography (SE-HPLC) and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Using this process, the proteins polymerize, forming intermolecular bonds among the same classes of proteins. Furthermore, the polymerization occurs only through disulphide bonds. Although SDS–PAGE did not show any differences for either the number or intensity of protein bands between flour samples before and after microwave treatment, gliadins from treated flours showed significantly reduced cross-reactivity with the R5 antibody. Moreover, the gluten became soluble in an aqueous saline solution, and it was not possible to isolate it using the Glutomatic apparatus. However, the treated flour, in the presence of water, was able to form dough and leaven and produce bread.

Keywords: Gluten proteins, Microwave treatment, R5 antibody, SE-HPLC, SDS–PAGE

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1. Introduction

Gluten is one of the earliest protein fractions described by chemists. It is defined as the ‘‘cohesive, visco-elastic proteinaceous material” that remains when wheat dough is washed to remove starch granules and water-soluble constituents (Shewry, 2009). Gluten contains hundreds of proteins, which are present either as monomers or as oligomers and polymers linked by inter-chain disulphide bonds (Shewry & Halford, 2002) and characterized by high contents of glutamine and proline and low contents of charged amino acids.

Traditionally, gluten proteins have been grouped according to their solubility in alcohol-water solutions, such as 60% ethanol, as the soluble gliadins and the insoluble glutenins (Osborne, 1924). The interchain disulphide bonds largely determine these properties, with the glutenin consisting of disulphide-stabilized polymers. Reduction of these inter-chain bonds allows the separation of the glutenin subunits into low molecular weight (LMW) and high molecular weight (HMW) groups. By contrast, the alcohol soluble gliadin fraction consists mainly of monomeric proteins, which either lack cysteine (x-gliadins) or contain only intrachain disulphide bonds (a-type and c-type gliadins). Gluten proteins are susceptible to heat treatment, and their behaviour under relatively high temperatures has been primarily evaluated in model systems. When glutenin is heated above 55 °C or gliadins are heated above 70 °C, disulphide/sulphydryl (SH) exchange reactions occur (Schofield, Bottomley, Timms, & Booth, 1983). Lavelli, Guerrieri, and Cerletti (1996) showed that 65 °C particularly influenced the S–S structure of HMW albumins and possibly their linkage to glutenin oligomers. Furthermore, LMW albumins and gliadins are affected at higher temperatures. In addition, Singh and MacRitchie (2004) found that glutenins polymerize below 100°C but that the polymerization of gliadins occurs only at higher temperatures. The molecular size of the glutenin aggregates increases, decreasing their extractability (Lamacchia et al., 2007; Schofield et al., 1983; Weegels, Verhoek, de Groot, & Hamer, 1994a). At 100 °C, gliadins undergo similar changes. The extractability of gliadins from bread, using 70% ethanol, is much lower than that of gliadins from flour, and a- and c-gliadins are more affected than are x-gliadins (Wieser, 1998). The effects have been ascribed to sulphydryl (SH)-disulphide interchange reactions induced by heat, which affect all gluten proteins except the cysteine-free x-gliadins (Schofield et al., 1983). Morel, Redl, and Guilbert (2002) suggested that, at temperatures below 60 °C, no changes in free sulphydryl groups occur. Heating to at least 90°C leads to disulphide bond linked aggregates and conformational changes, primarily affecting gliadins and low-molecular-weight

albumins and globulins (Guerrieri, Alberti, Lavelli, & Cerletti, 1996). Although, Kokini, Cocero, Madeka, and de Graaf (1994) proposed that cross-links among gliadin molecules are formed above 70°C in the absence of glutenins, others have hypothesized that gliadins cross-link only with glutenins

(Redl, Morel, Bonicel, Vergnes, & Guilbert, 1999; Singh & MacRitchie, 2004) and that the incorporation of gliadin monomers in the glutenin network leads to a three-dimensional structure (Morel et al., 2002). In a study performed on pasta, Lamacchia et al. (2007) showed that gluten proteins undergo changes induced by drying cycles, were consistent with the results found in the model system. Furthermore, the albumins and globulins were completely denatured at 60°C and incorporated into high-molecular-weight polymers, whereas, because of the conformational structure and low thioavailability, gliadins were only partially denatured at 90°C and aggregated into polymers of higher molecular weight. In a recent study, Lamacchia, Baiano, Lamparelli, La Notte, and Di Luccia (2010) reported that high temperature applied to durum wheat kernels induced significant changes in proteins but these were different from those reported in a gluten model system and pasta. In particular, proteins were denatured and polymerized, and the albumins and globulins tended to coagulate and interact with gliadins instead of HMW glutenins to form protein aggregates of intermediate molecular weight, revealed as a new intermediate protein peak. The explanation for this phenomenon was that, in the kernel, gluten is not formed and gluten proteins are deposited in different protein bodies (Rubin, Levanony, & Galili, 1992). Tosi, Gritsch, He, and Shewry (2011) confirmed that, in wheat kernels, gluten proteins may form protein bodies by two mechanisms, either transport via the Golgi and Golgi-derived vesicles into the vacuole or by direct accumulation within the lumen of the ER. Additionally, the segregation of protein types into specific populations of protein bodies can occur within the same cell. Differences in the proportions of different types of gluten proteins in protein bodies within the same cell was also demonstrated by immunofluorescence, using double antibody labelling. Therefore, cells of the central starchy endosperm clearly contained protein bodies, which contained HMW subunits of glutenin but little or no gliadin, and this segregation may be maintained, even after protein body fusion and matrix formation. Apart from fundamental interests, the effects of heating gluten proteins are relevant to practical processes, such as the drying of rain-damaged wheat, drying of gluten from starch/gluten manufacturing plants, and in relationship to effects on gluten proteins during baking and extrusion. Because Lamacchia et al. (2010) showed, for the first time, that the application of high temperature to wheat grains generates protein polymerization different from that reported in a gluten model system or in bread or dried pasta, the effects of heating gluten proteins in wheat grain have potential for producing modified gluten with unique properties. In the present study, we examined the behaviour of gluten proteins in wheat kernels subjected to high temperature for a short time, using a high percentage of seed moisture and microwave energy (Italian Patented Method N. 0001414717, 2015; Patent Cooperation Treaty n. PCT/IB2013/000797, 2013). The polymerization of the different protein classes was ascertained, as were the changes in the glutenin and gliadin fractions separated by SDS– PAGE. In addition, the

effects of changes in the protein fractions induced by the microwave treatment on both the binding ability of the R5 antibody and the bread-making properties were also evaluated.

2. Materials and methods

2.1. Raw materials and microwave treatment

The wheat kernels (Blasco, Adamello, Ofanto, and Simeto varietie and mixtures of soft and durum wheat Canadian grains) used in this study were supplied by CRA (Foggia, Italy) and by the Casillo group S.p.a (Corato, Italy), respectively. Treated wheat flour (TWF) was obtained by milling the microwave-treated caryopses (Italian Patented Method N.0001414717, 2015; Patent Cooperation Treaty n. PCT/IB2013/000797, 2013) that were previously harvested and threshed. In particular, 100 g of cleaned wheat grains were dampened dampened for at least two hours, until reaching 18–20% humidity, and then measured with a halogen thermal balance (Mettler Toledo, HB43-S, Swiss). These were then drained and subjected to 1000 watt power for 2 min in a microwave apparatus (Delonghi, Italy) to reach a temperature of approximately 110–120 °C and then measured with a thermal camera (FLUKE i 20 model, Italy).

These parameters were set on the basis of the work of Lamacchia et al. (2010), who induced polymer protein changes but eliminated the negative effects due to the burning of the caryopses. After microwave treatment, the wheat kernels were cooled and dried at room temperature (24°C) for 12–24 h and then ground, using a roller mill. The particle size of the TWF used was in the range of 100–200 μm. Experiments on TWF were performed with the flour obtained from wheat kernels before the microwave treatment as the control wheat flour (CWF). For each grain sample, (Adamello, Blasco, Ofanto, Simeto, mixture of Canadian durum wheat grains and mixtures of Canadian soft wheat grains), three independent experiments were performed.

2.2. Proteins extraction for SE-HPLC

Proteins from the CWF and TWF samples were extracted, using the method of Gupta, Khan, and MacRitchie (1993). Soluble proteins from 10 mg of samples were extracted with 1 ml of 0.5% SDS–phosphate buffer (pH 6.9). The suspension was shaken for 30 min and the solubilized protein (‘‘soluble” or ‘‘extractable” protein) was recovered by centrifugation for 10 min. The resulting residues were extracted with 1 ml of 0.5% SDS phosphate buffer (pH 6.9) by sonication for 15 s (Microson Ultrasonic cell distributor), ensuring that the samples were completely dispersed within the first 5 s, and then heated to 35 °C for 30 min. The supernatants after centrifugation (10 min at 17,000g) were named ‘‘unextractable” proteins or ‘‘insoluble” proteins. Total proteins (10 mg) were extracted in 1 ml of the same buffer, vortexed, and sonicated for 30 s, and the supernatants (‘‘total”

protein) were recovered for SE-HPLC analysis. All extracts were filtered through a 0.45 μm PVDF filter prior to injection on the column.

2.3. SE-HPLC analysis

SE-HPLC was performed, using an LC Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) and Phenomenex SEC S-4000 column (300 x 7.8 mm, Phenomenex, Torrence, CA, USA). Each sample (20 μl) was injected onto the column, and the
eluted proteins were monitored at 214 nm. Three replicates of each sample were used to investigate the protein composition. The mobile phase was 50% acetonitrile containing 0.05% trifluoroacetic acid with a flow rate of 0.7 ml/min. The SE-HPLC column was calibrated, using protein standards with a range of molecular weights (kDa) as follows: ribonuclease A (13.7), chymotrypsinogen (25.0), ovalbumin (43.0), bovine serum albumin (67.0), aldolase (158), catalase (232), ferritin (440) and thyroglobulin (669).
The percentage of unextractable polymeric protein (UPP) was calculated as described by Gupta et al. (1993). Briefly, the percentage of total UPP was calculated as [peak LPP (Large Polymeric Proteins) + SPP (Small Polymeric Proteins) area (unextractable)/peak LPP + SPP area (total)] 100. Peak LPP + SPP area (total) refers to the total of peak LPP + SPP (extractable) and peak LPP + SPP (unextractable) (Kuktaite, Larsson, & Johansson, 2003).

2.4. Determination of SH and S–S groups

The protein disulphide and sulphydryl contents in the CWF and TWF samples were estimated by colorimetric determination of the free SH groups, using the solid phase assay with $NTSB^{2-}$ according to the method of Chan and Wassermann (1993).

2.5. SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) analyses

Total proteins from the samples (1 g) were extracted using 10 ml of an extraction buffer containing 0.0625 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol (v/v), and 5% dithiothreitol (DTT) (w/v). CWF and TWF samples were incubated in the extraction buffer for 2 h and were then centrifuged at 9500 rpm for 15 min at 10 _C. The supernatants containing proteins were carefully removed and stored at _20 _C until used. Gliadins and glutenins from flours (1 g) were extracted, using the Osborne sequential extraction method (Osborne, 1924).

To separate the extracted proteins, SDS–PAGE was performed on a 12.5% gel under reducing and non-reducing conditions, using a vertical electrophoresis system, Hoefer SE 600 (GE Healthcare, Milan, Italy). SDS–PAGE analysis was performed at 25 mA for 3 h at room temperature. The gels

were stained with 0.25% w/v Coomassie Brilliant Blue (CBB) overnight. Gel images were taken, using a Chemi-Doc imager (Chemidoc MP, Bio-Rad Laboratories). Quantification of the bands was carried out, using image analysis software (Phoretix Total Lab, Phoretix International).

2.6. Gluten antigenicity assessment

An R5-sandwich ELISA analysis (Valdés, Garcìa, Llorente, & Méndez, 2003) of the CWF and TWF samples was performed by the Istituto di Ricerche Agrindustria (Modena, Italy). Samples were treated in accordance with the Ridascreen_Gliadin ELISA kit (RBiopharm AG Darmstadt, Germany) which is a sandwich enzyme immunoassay for the quantitative analysis of contaminations by prolamins from wheat (gliadin), rye (secalin), and barley (hordein) in raw products, such as flours (buckwheat, rice, corn, oats, teff) and spices, as well as in processed food and heat-processed wheat- and barley-based products. This kit is able to identify gliadins,
hordeins and secalins with assay sensitivities of 0.78, 0.39 and 0.39 ng/ml, respectively. The assay's detection limit was 1.5 ng gliadins/ml (1.56 ppm gliadins, 3.2 ppm gluten). For gliadin
extraction, a 25 mg sample was dispersed in 2.5 ml of Cocktail Solution (Art. No. R7006, official R5-Mendez method), provided by the Ridascreen Gliadin Kit, for 40 min at 50 °C and then extracted with 80% ethanol, mixed for 1 h at room temperature and centrifuged at 3000g for 10 min at room temperature. The supernatant was diluted with sample diluent and 100 µl of diluted samples or standards were added to each separate duplicate well pre-coated with gliadin antibody. After 30 min of incubation at room temperature, the liquid was poured out and the plate was tapped upside down against absorbent paper. Then the plate was washed three times with washing buffer (R-Biopharm AG Darmstadt, Germany) to remove unbound antigen. Thereafter, 100 µl of diluted horse radish peroxidase (HRP) conjugated detecting antibody (R-Biopharm AG Darmstadt, Germany) were added to each well, which were incubated at room temperature for 30 min followed by three times washing. Then the wells were filled with 50 µl of substrate and 50 µl of chromogen (R-Biopharm AG Darmstadt, Germany), and incubated for 30 min at room temperature in the dark to develop the colour. The reaction was stopped by adding 100 µl of stop solution (R-Biopharm AG Darmstadt, Germany), to each well and absorbance was measured with a microplate reader (SEAC, Sirio model, Calenzano, Florence, Italy). The antigenicity was calculated from the standard curve, by using the Ridasoftwin software (R-Biopharm AG Darmstadt, Germany), as equivalent mg of gluten/kg of sample. The cocktail extraction procedure for heat-processed food samples has the advantage that aggregated a- and c- fractions are solubilized and extracted and can still react specifically with R5 antibody.

2.7. Gluten index and baking process

The gluten index was determined according to the ICC procedure 137/1. Bread was baked as ‘‘Francesini” or ‘‘Ciabatta” from the CWF and TWF samples in a bakery (LA.PA s.r.l., Crema, Italy). Dough was prepared by mixing all ingredients in a dough mixer as follows: 1.5 kg of flour, 5% sourdough, 57% water and 1.8% salt. The doughs were leavened for approximately 20 min (at 28/30 °C and 70% humidity) and then shaped into either ‘‘Francesini” or ‘‘Ciabatta” and leavened again for 40 min. The leavened bread was placed on Teflon perforated trays and baked in an oven at 220 °C for 20 min.

2.8. Statistical analysis

Data are reported as the means of three replicates. Mean differences were compared by unpaired Student’s t-test. The statistical packaged SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) was used. A p-value <0.05 was considered significant.

3. Results and discussion

3.1. Effect of microwave treatment on flour glutenin polymers

The proportion of large glutenin polymers is an important determinant of end-use quality because it relates directly to gluten and dough elasticity (strength) (Gupta, Masci, Lafiandra, Bariana, & MacRitchie, 1996). Size-exclusion (SE)-HPLC was used to determine changes in the glutenin polymer size distribution after the microwave treatment described in Section 2.1.

The method uses sonication in 2% (v/v) SDS solution to render all glutenin polymers soluble and within the size fractionation range for SE-HPLC. During the extraction, some cleavage of the polymers occurs and the size distribution observed is therefore not identical to that of the native polymers. However, the procedure is highly reproducible, and good correlations between the polymer size distribution and functional properties are observed (Gupta et al., 1993). A typical size-exclusion profile (Fig. 1) shows some peaks corresponding to the gluten protein fractions (called LPP, SPP and LMP in Fig. 1) and additional peaks that correspond to other components (SMP in Fig. 1). Analysis of these fractions by SDS–PAGE showed that LPPs (large polymeric proteins) consist mainly of high-molecular-mass polymers enriched in HMW subunits of glutenin and that SPPs (small polymeric proteins) are mainly low molecular- mass glutenin polymers. LMPs (large monomeric proteins) consist mainly of x-, a-, b- and c-type gliadins (Morel, Dehlon, Autran, Leygue, & Bar-L’Helgouac’h, 2000), and SMPs (small monomeric proteins) are mainly non-gluten proteins and low-molecular-mass components. The sums of LPP + SPP + LMP + SMP and LPP + SPP + LMP provide an estimate of the total proteins and total gluten proteins, respectively. The SE-HPLC charts for the samples before (CWF) and after microwave treatment (TWF) are shown in Fig. 1. Table 1 shows the change

in the amount of total protein, which was measured as the chromatogram area and the two area ratios %LPP/%SPP and %LMP/%LPP for the two different treatments. The proportion of large polymers LPP may correlate with dough strength but the ratios of %LPP/%SPP and %LMP/% LPP usually show stronger correlations (Millar, 2003). There are no clear differences in the profiles (Fig. 1) of the two samples (CWF vs TWF), whereas significant changes are detected lower (110–120 °C) than that used in the previous study (Lamacchia et al., 2010) in which the seeds were arranged on a steel bearing placed into direct contact with a wood-fire for 120 s until the kernel browned. The decrease of total peak area indicates that microwave treatment reaches high temperatures and denatures protein fractions, making them unextractable, or allows the polymerization of proteins in larger polymers that become unextractable (Lagrain, Brijs, Veraverbeke, & Delcour, 2005). The ratios of %LPP/%SPP and %LMP/%LPP, beside the correlation with dough strength, allow also, for a better understanding of which fraction of gluten protein was more susceptible to the microwave treatment. The significant decreases in the %LPP/%SPP value and the increase in the %LMP/% LPP, respectively, indicate that, among the different classes of proteins, the large polymeric proteins, LPPs, were more denatured or polymerized.

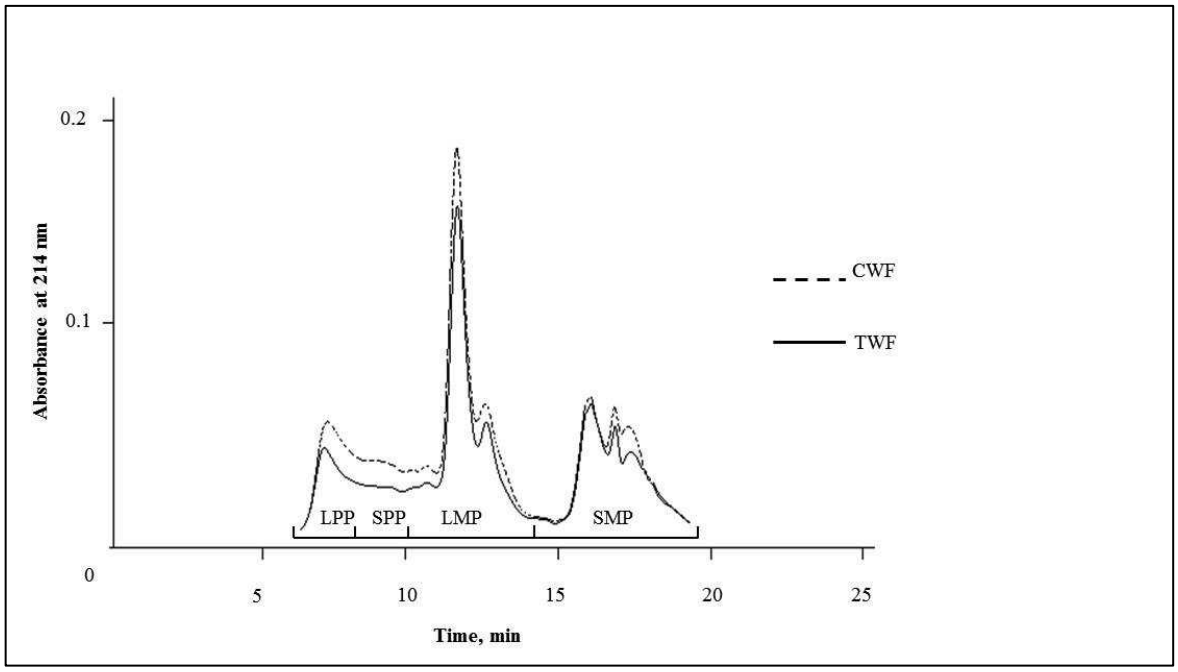

Fig. 1. SE-HPLC elution profile of total proteins (‘‘extractable” and ‘‘unextractable”) of CWF (control wheat flour) and TWF (treated wheat flour) of the Blasco variety, representative of the other wheat varieties and wheat grain mixtures, before and after the microwave treatment described in this study. LPP: large polymeric proteins; SPP: small polymeric proteins; LMP: large monomeric proteins; SMP: small monomeric proteins.

Samples	Total peak areas (mm^2)	LPP/SPP (%)	LMP/LPP (%)
CWF	9349 A	29.13 A	1.55 A
TWF	7112 B	22.29 B	2.36 B

A and B = $P < 0.001$

Table 1. Values of the total peak areas for the investigated CWF (control wheat flour) and TWF (treated wheat flour) samples computed from SE-HPLC profiles of ‘‘total” proteins (‘‘extractable and ‘‘unextractable”). LPP/SPP% ratio and LMP/LPP% ratio: differences between CWF and TWF. The data are reported as the mean values ± standard deviation (n = 3 independent experiments for each grain sample used; Blasco, Ofanto, Adamello, Simeto, mixtures of Canadian durum wheat grains and mixtures of Canadian soft wheat grains). Different letters indicate significant differences at $P < 0.01$ (capital letters) and $P < 0.05$ (small letters).

This is consistent with the observation that glutenins (LPP and SPP) are susceptible to an increase in temperature and that glutenin denaturation is complete at 60°C, whereas gliadins (LMP), because of their conformational structure, are more stable to the increase in temperature, and denaturation begins at 60 °C and increases significantly at 90 °C (Lamacchia et al., 2007; Schofield et al., 1983; Singh & MacRitchie, 2004). The proteins with the highest molecular weight have the highest correlation with strong dough properties (MacRitchie, 2014). A certain amount of these polymers remains unextractable in various extracting systems (acetic acid solution or sodium dodecyl sulphate phosphate buffer). The %UPP (percentage unextractable polymeric protein in total polymeric protein) is often used as a measurement of the amount and size distribution of the polymeric protein (Gupta et al., 1993). A high %UPP value is related to a greater proportion of glutenin that is insoluble in sodium dodecyl sulphate without sonication and is likely associated with the highest molecular weight (Singh & MacRitchie, 2004). Therefore, wheats with a greater percentage of UPP, are expected to have a greater dough resistance (elasticity) and a longer mixing requirement than have those with a greater proportion of extractable polymeric proteins (Gupta et al., 1993). Because the amount of UPP is influenced by different quaternary structures, which result from polymers involving disulphide bridges and strong crosslinking bonds through dityrosine formation (Tilley et al., 2001) and aggregates involving hydrogen bonding (Aussenac, Carceller, & Kleiber, 2001), both intra- (formed by gliadins and glutenins) and inter- (formed by glutenins) chain disulphide bonds, as well as non-covalent bonds, are predicted to be important for the formation of the gluten polymer complex. Table 2 shows the proportion of UPP and total cysteine and S–S bonds of the grains before and after microwave treatment. The percentage of total UPP in the treated sample decreases significantly, consistent with the SE-HPLC chart and with the two area ratio values, but a concomitant increase in the S–S bonds is also detected. These results suggest that this microwave treatment induces modification in the SH and disulphide bonds, moving polymeric proteins, and in particular, the HMW (LPP) subunits towards larger insoluble protein aggregates, which become unextractable, even after sonication. This is consistent with the conclusion reached by Singh and MacRitchie (2004). In

addition, Table 2 shows an increase in the total cysteine, suggesting that microwave treatment induces a major exposure of the cysteine residues due to the dissociation and unfolding of proteins.

Samples	UPP (%)	S-S concentration (µmol/g of protein)	SH-free concentration (µmol/g of protein)	Total cysteine concentration (µmol/g of protein)
CWF	37.3 A	51.38 ± 0.72 A	71.67 ± 0.39 A	174.45 ± 1.66 A
TWF	28.4 B	78.05 ± 0.48 B	66.34 ± 0.92 B	222.45 ± 1.68 B

A and B = $P < 0.001$

Table 2. Percentages of total UPPs (unextractable polymeric proteins), SH-free (total free cysteines), S–S (disulphide bond) and total cysteine concentration. Differences between the CWF and TWF samples. Data are reported as the mean values ± standard deviation (n = 3 independent experiments for each grain sample used as follows: Blasco, Ofanto, Adamello, Simeto, mixtures of Canadian durum wheat grains and mixtures of Canadian soft wheat grains). Different letters indicate significant differences at $P < 0.01$ (capital letters) and $P < 0.05$ (small letters).

In fact, the modification of proteins by heat usually results in changes in the secondary and tertiary structure of the protein molecules that become denatured. The process of denaturation involves the dissociation and unfolding of proteins and is often accompanied by the formation of disulphide linkages and the exposure of hydrophobic amino acids on the surface. The degree of heat treatment results in differences in the extent of the denaturation and functionality of the proteins. The more the hydrophobic amino groups are exposed, the more insoluble the proteins become.

3.2. Effect of microwave treatment on flour protein fractions

To better understand this phenomenon, total proteins (Fig. 2A) and the glutenin and gliadin fractions (Fig. 2B) of CWF and TWF were separated by SDS–PAGE under reducing and non-reducing conditions. Analysis of these fractions by SDS–PAGE (Fig. 2) showed that the microwave treatment did not cause either a decrease in the number of bands or a decrease in the intensity of the bands. These results, confirmed by quantification of band intensities obtained via image analysis, suggest that TWF proteins are not denatured, do not interact with other compounds in wheat endosperm and that the decrease of the total peak area observed by SE-HPLC analysis is primarily due to protein polymerization that occurs only through disulphide bonds. These last results are consistent with the work of Weegels et al. (1994a) and Weegels, de Groot, Verhoek, and Hamer (1994b) but are in contrast to the work of Guerrieri and Cerletti (1996). Guerrieri et al. (1996) and Lamacchia et al. (2010) suggest that, at temperatures higher than 100 °C, changes other than disulphide bridging are involved.

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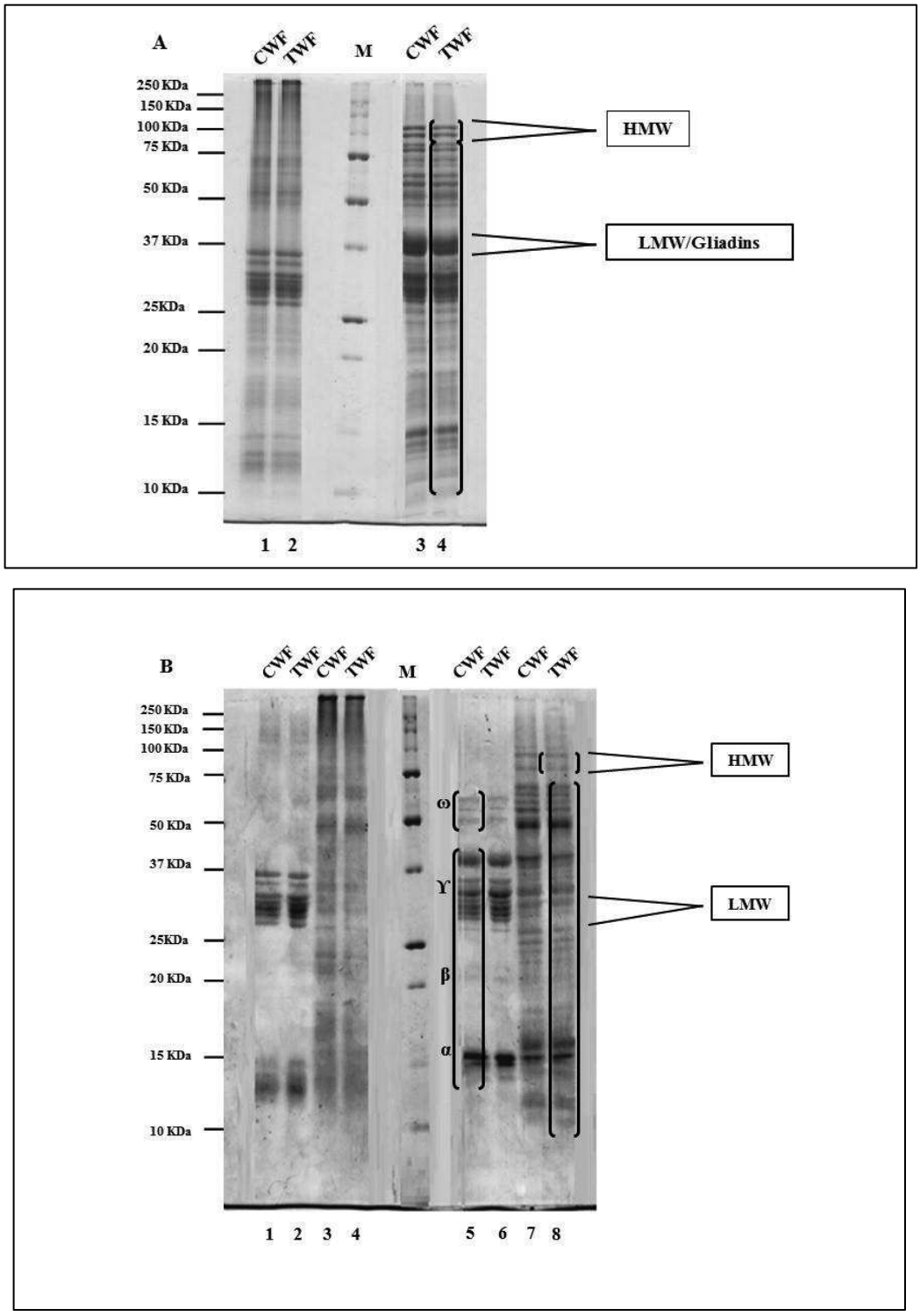

Fig. 2. SDS–PAGE separation of CWF (control wheat flour) and TWF (treated wheat flour) of the Blasco variety, representative of the other wheat varieties and wheat grains mixtures, before and after microwave treatment described in this study. (A) Total protein under non-reducing (1–2) and reducing (3–4) conditions. M: marker. (B) Protein fractions under non-reducing (1–4) and reducing (5–8) conditions. Lane 1: CWF gliadins; lane 2: TWF gliadins; lane 3: CWF glutenins; lane 4: TWF glutenins; lane 5: CWF gliadins; lane 6: TWF gliadins; lane 7: CWF glutenins; lane
8: TWF glutenins M: marker.

CWF and TWF gliadins were tested for their ability to bind a specific monoclonal antibody, R5, using the high sensitivity of the R5-ELISA (Valdés et al., 2003), which is used to monitor residual gluten levels as low as 3.2 ppm in all commercial gluten-free foods. Interestingly, the R5-ELISA indicated a significant reduction (99%) in the levels of detectable gluten protein, showing that with this microwave treatment, gliadin significantly decreases its cross-reactivity with the R5 monoclonal antibody. In particular, the amount of gluten detected decreased from 10–12% (CWF) to 60 ppm ± 3 (TWF) in durum wheat grains and to 40 ppm ± 3 (TWF) in soft wheat grains. The R5-ELISA, used in this work, includes an extraction procedure allowing aggregates of gliadins generated by the heat-process to be solubilised and it is well known that heat treatment leaves QQPFP epitopes unchanged (Valdés et al., 2003). The R5 antibody also recognizes the epitopes-LQPFP, QLPYP and PQPFP in the sequence of the recently described potent inducer of gut-derived human T-cell lines in coeliac patients, the A gliadin 33-mer peptide of residues 57–89 (LQ LQPFPQPQLPYPQPQLPYPQPQL–PYPQPQPFP) (Arentz-Hansen et al., 2000). The reduced gluten antigenicity observed with the R5-Elisa is in accordance with preliminary results showing that flours treated by this patented method did not induce production of the inflammatory cytokine, interferon gamma, when tested, after digestion and deamidation, on gut-derived human T-cell lines of celiac patients highly reactive to 33-mer, omega and gamma peptides (Lamacchia, Di Luccia, & Gianfrani, 2015). To the best of our knowledge, only Kwak et al. (2012) have tried to reduce the antigenicity of gliadins in medium wheat dough by using microwave energy at 1000 watts for 1, 5 and 10 min. The results showed that the microwave treatment did not reduce the antigenicity of wheat gliadins, which was measured as the ability of anti-gliadin IgG to bind gliadins, and that the antigenicity increased as the time of microwave exposure and the degree of protein denaturation increased. It can be argued that the denaturation of wheat proteins and the increase of antigenicity during microwave treatment occur after the gluten is already formed. For wheat proteins subjected to microwave energy while present in the endosperm, denaturation of the different protein classes occurs minimally and may be caused by their deposition in different protein bodies in their native form, allowing for chemical changes otherwise impossible in the already formed gluten. This dramatically reduces the antigenicity of gluten.

3.3. Effect of microwave treatment on gluten index (GI) and breadmaking properties of wheat flours

The gluten index (GI) is a measure of the gluten characteristics, which indicates whether the gluten is weak, normal or strong. Gluten is separated from wheat flour using the Glutomatic equipment, taking advantage of the insolubility of gluten proteins in aqueous saline solutions (Osborne, 1924). Next, gluten is centrifuged to force the wet gluten through a specially constructed sieve under

standardized conditions. The total weight of gluten is defined as the gluten quantity. The percentage of wet gluten remaining on the sieve after centrifugation is defined as the gluten index. If the gluten is very weak, all of the gluten may pass through the sieve, and the gluten index is 0. When nothing passes through the sieve, the index is 100. CWF and TWF samples were analysed for GI and results showed that the isolation of gluten from the TWF samples was not possible. Consequently, it was also not possible to calculate a GI to determine its strength. These results suggest that microwave treatment induces significant modification of gluten proteins such that they become soluble in aqueous solution, although these modifications are not observed in the SDS–PAGE analysis (Fig. 2A and B). The main reasons for the water-insoluble characteristics of gluten are the hydrophobic nature of its proteins, consisting of a large percentage of uncharged amino acid residues (glutamines Gln; asparagines Asn) and the presence of hydrogen bonds. By transforming the amides of Gln and Asn into carboxyl groups through deamidation, the solubility of proteins in wheat gluten is transformed with effects such as changing of the charge density, increasing the electrostatic repulsion and breaking the hydrogen bonds (Liao et al., 2010; Riha, Izzo, Zhang, & Ho, 1996). In our case, the solubility of gluten proteins cannot be due to the deamidation of amino acids because no difference between the CWF and TWF proteins bands was detected during the SDS–PAGE separation (Fig 2A and B).

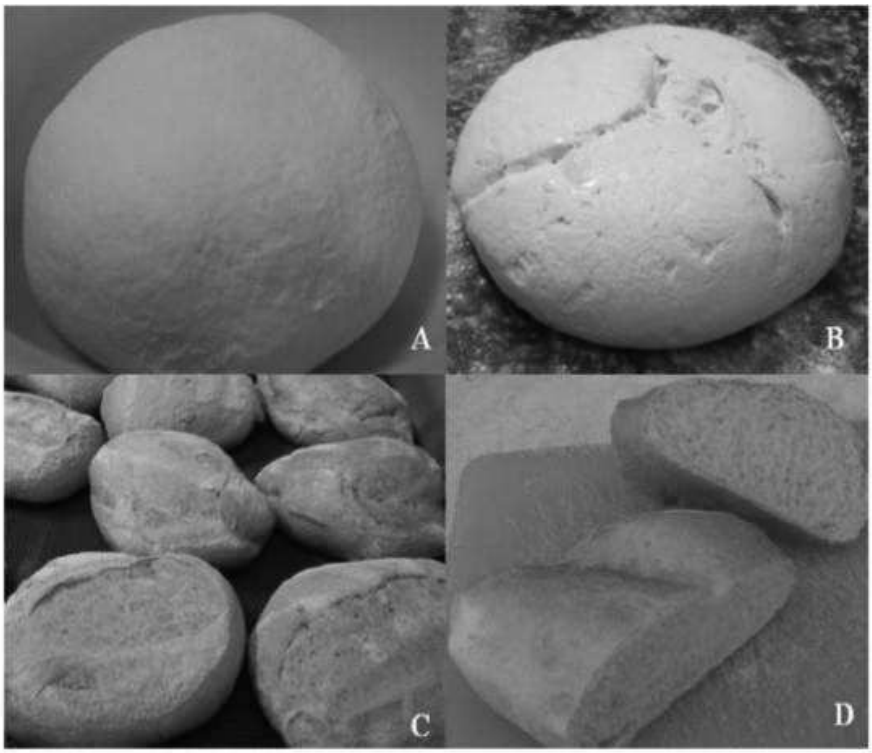

Fig. 3. Bread-making properties of TWF (treated wheat flour) samples. (A) Dough before leavening. (B) Dough after leavening. (C) Bread baked as ‘‘Ciabatta”. (D) Bread baked as ‘‘Francesino”. Dough and breads in the picture were obtained from a mixture of Canadian soft wheats treated by the microwave process described in this study and are representative of the other treated grain samples.

However, high temperature for short time, generated by the microwave treatment applied to the caryopses, may break the hydrogen bonds between protein glutamine residues (Shewry, Popineau, Lafiandra, & Belton, 2001) when proteins are in their native form in protein bodies, allowing conformational and/or structural changes (Liao et al., 2010), as evidenced by the increase in the total cysteine levels (Table 1). This would explain the significant decrease in gliadin cross-reactivity towards the R5 monoclonal antibody because it recognizes the repetitive pentapeptide QQPFP epitope.

Because the GI analysis did not allow for gluten separation for the TWF samples and because gluten is the proteinaceous mass that confers cohesive and visco-elastic properties to dough, allowing the production of bread, baked goods and pasta, bakery tests were performed to understand the degree of gluten protein modification. Breads were baked in a bakery (LA.PA s.r.l, Crema, Italy), and although deep modification of the gluten properties was detected using the Glutomatic analysis, the TWF samples absorbed 10% more water than do the CWF samples when forming a dough (Fig. 3A), which preserves most of the plastic characteristics of the gluten, allowing the leavening and production of bread (Fig. 3B–D). TWF breads showed all of the characteristics of the control bread (CWF); however, a decrease in the loaf volume was detected and expected because of a decrease of dough elasticity. The elasticity of dough is due to HMW glutenin subunits and, in particular, to the regions associated with its b-turn structure that is rich in glutamines and proline (Shewry et al., 2001). Numerous studies suggest that hydrogen bonds between glutamines of glutenin subunits and polymers may also contribute to elasticity (Shewry et al., 2001), as shown in a new model, which is now termed the ‘‘loop and train” model (Belton, 1999). The decrease in the elasticity of the TWF samples is consistent with the observation that breaking the hydrogen bonds between gluten proteins and conformational changes induced by microwave treatment in the kernel allow polymers in the flour to be hydrated but not to form a high amount of loop to train structures that are important for determining dough elasticity. Furthermore, breaking the hydrogen bonds between protein glutamines in wheat kernels induced by microwave treatment may be consistent with the increase in the percentage of water adsorbed by the flour during mixing. In fact, if more glutamines are available to form hydrogen bonds with water for the plasticization of dough, a higher percentage of water must be added to the flour.

4. Conclusions

The microwave treatment used in this study and applied in hydrated wheat kernels reaching a high temperature for a short time induced significant changes in gluten proteins. Among these changes, the most important are the reduced antigenicity (by 99% in comparison with the control samples) and

the solubility of gluten in saline aqueous solution. These changes suggest that the microwave treatment applied to wheat kernels may allow the breaking of hydrogen bonds between glutamine residues when proteins are in their native form in protein bodies, thus favouring protein conformational and/or structural changes. The present study, for the first time, shows the mechanism by which a chemical-physical treatment abolishes the antigenic capacity of gluten such that it is unrecognizable by the antibody used worldwide to determine the amount of gluten in flour for celiac patients and also shows how this modification allows flour to be processed to make bread. As proposed by the Codex Alimentarius Committee, the flour described in this study should be classified as a very low gluten content (21–100 ppm of gluten) for use in the production of food with superior sensory properties for the diet of patients with gluten-correlated disorders (intolerant and sensitive to the gluten) or for those genetically predisposed to this pathology. However, it is subject of further investigation to understand both the molecular changes occurring and how these changes affect digestibility, availability and characterisation of potential antigenic fragments postdigestion. Further studies should also focus on the reactivity of the products in gluten-sensitive patients by more complex immunological and clinical trials.

Acknowledgment

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Chapter 3

Impact of gluten-friendly™ technology on wheat kernel endosperm and gluten protein structure in seeds by light and electron microscopy

Impact of gluten-friendly^{TM} technology on wheat kernel endosperm and gluten protein structure in seeds by light and electron microscopy

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Abstract

The main aim of this paper was to assess the impact of Gluten-FriendlyTM (GF) technology (Italian priority patent n_ 102015000084813 filed on 17th December 2015) on wheat kernel endosperm morphology and gluten protein structure, using SEM, light and immunofluorescent microscopy. Microscopy was combined with immunodetection with specific antibodies for gliadins, c-gliadins, LMW subunits and antigenic epitopes to gain a better understanding of the technology at a molecular level. The results showed significant changes to gluten proteins after GF treatment; cross-reactivity towards the antibodies recognizing almost the entire range of gluten proteins as well as the antigenic epitopes through the sequences QQSF, QQSY, PEQPFPQGC and QQPFP was significantly reduced. The present study confirms the results from our previous work and shows, for the first time, the mechanism by which a chemical-physical treatment abolishes the antigenic capacity of gluten.

Keywords: Gluten-friendly, SEM, Immunofluorescent microscopy, Antigenic epitopes

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1. Introduction

Wheat endosperm contains 8–15% protein, 80% of which is made up of gluten. Gluten consists of aqueous alcohol-soluble gliadins and insoluble glutenins. Gliadin is a mixture of heterogeneous monomeric proteins, divided into a/b, c and x-gliadins. Both a/b and c-gliadins are low-molecular-weight proteins (MW 28– 35 kDa) with six and eight cysteine residues, respectively, whereas x-gliadins (MW 40–75 kDa) do not contain cysteine. Glutenin is a polymeric protein composed of high-molecular-weight (HMW-GS, MW 65–90 kDa), and low-molecular-weight glutenin subunits (LMW-GS, MW 30–45 kDa) linked by inter-chain disulphide bonds (Wieser, 2007). Gluten's unique physico-chemical properties make wheat suitable for preparing a wide range of food products, including breads, noodles, pastas and biscuits. Although gluten is used extensively in food products, it has been associated with coeliac disease (CD) in some individuals. Coeliac disease is a chronic immune-mediated enteropathy triggered by the ingestion of gluten in HLA-DQ2- or HLA-DQ8-positive subjects, mainly resulting in small-intestinal mucosal injury and nutrient malabsorption in susceptible individuals (Rossi & Schwartz, 2010). Approximately 30% of the general population carry the HLA-DQ2/8 coeliac disease susceptibility genes; however, only 2–5% of these individuals will go on to develop coeliac disease, suggesting that additional environmental factors contribute to disease development (Rossi & Schwartz, 2010). The only effective treatment available for CD patients is strict exclusion of gluten from their diet. The detrimental consequences of consuming gluten and/or analogous proteins (present in rye, barley and oats) are well-documented, showing that noncompliance with a gluten-free diet is associated with increased risk of anaemia, infertility, osteoporosis and intestinal lymphoma.

An alternative way to exclude gluten is to detoxify it without affecting the technological performances of the resulting flour and dough. Recently, we have developed a new and innovative detoxification method of gluten proteins from cereal grains (Italian patented method n_: 0001414717, also filed under the Patent Cooperation Treaty, application no. PCT/IB2013/000797) (Lamacchia, Di Luccia, & Gianfrani, 2013, 2015a), aiming to combine the nutritional and technological properties of wheat proteins with safety for coeliac sufferers and other gluten-sensitive subjects.

This innovation is usually referred to as ‘‘Gluten FriendlyTM" and relies on 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 and induce a structural change in gluten proteins (Lamacchia, Landriscina, & D'Agnello, 2016). This modification abolishes the antigenic capacity of gluten (Lamacchia et al., 2016) and reduces the immunogenicity in vitro of the most common epitopes involved in coeliac disease (Lamacchia, Di Luccia, & Gianfrani, 2015b), without compromising the

nutritional or the technological properties necessary to process semolina in pasta and flours in bread and other baked goods

(Lamacchia et al., 2016). Proteins are present in the wheat in their native form, as they are located in protein bodies, thus preventing microwave treatment from causing protein denaturation. However, the treatment is able to reduce gluten's antigenicity and this effect has been attributed to a structural change in the protein (Lamacchia et al., 2016). Moreover, Gluten FriendlyTM bread can also partly correct and positively modify the quali-quantitative composition of the microbiota of coeliac people in a model system (Bevilacqua, Costabile, et al., 2016). However, no data are available on the change occurring in grain seeds after Gluten FriendlyTM processing. Therefore, this paper addresses the effect of Gluten- FriendlyTM technology on wheat endosperm morphology and gluten protein structure in seeds, by SEM, light and fluorescence microscopy.

Immunodetection experiments with specific antibodies for gliadins, c-gliadins and LMW subunits were carried out to gain a better understanding of the technology at a molecular level and the mechanism by which it abolishes the antigenic capacity of gluten.

2. Materials and Methods

2.1 Raw materials and microwave treatment.

The wheat kernels (mixtures of soft wheat Canadian grains) used in this study were supplied by the Casillo group S.p.A. (Corato, Italy). Grains treated with microwaves were called Gluten-Friendly Grains (GFG) and were obtained by treating the caryopses, previously harvested and threshed, with microwave energy (Italian patented method n°: 0001414717 also filed under the Patent Cooperation Treaty, application no. PCT/IB2013/000797). The technology has since been 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 were dampened to 15-18% moisture; moisture was evaluated using a Halogen Moisture Analyzer (Mettler Toledo HB43-S, Switzerland). The seeds were heated with microwaves (DeLonghi, Italy, for about 1 min. between 1000 and 750 watt), followed by a phase of slow evaporation of the water content. Rapid heating and slow evaporation was 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 wheat kernels were cooled and dried at room temperature (24 °C) for 12-24 h. Seeds were left at room temperature until use. Grains before treatment were considered Control Grains (CG).

2.2 SEM (Scanning Electron Microscopy) assays

Freeze-fracture analyses were performed by dropping seeds (six per treatment, CG and GFG, respectively) into liquid nitrogen for thirty seconds and a single, sharp tap was administered to the grain in order to produce a fracture. Samples were then sputter-coated with gold and imaged in the FEI Quanta FEG 600 Environmental Scanning Electron Microscope equipped with a Quorum PP2000T Cryo Stage.

For Critical-Point drying (CPD) analysis, the grains (six per treatment, CG and GFG, respectively) were fixed for 3h in 2.5% glutaraldehyde in 25 mM HEPES buffer at pH 6.9, 4°C, and then rinsed in HEPES buffer and distilled water for 15 min. The specimens were then dehydrated through a 10% ethanol series at room temperature (10% to 100% ethanol series) and dried in the critical-point dryer (31°C/ 73.8 bar). Samples were then sputter-coated with gold and imaged in the JEOL JSM-6360 LV using a dry stage setting at a high voltage of 20.0 kV.

For immunogold analysis, freeze-fractured grains (five per treatment, CG and GFG, respectively) were fixed in formalin vapour for 96 hours by placing them in sealed Petri dishes containing a smaller open dish with 1 ml formalin. Grain pieces were stored in a desiccator containing silica gel until use, to prevent absorption of water from the air. Grains were briefly rinsed in PBS (Phosphate-buffered saline: 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) and then blocked in a 3% (w/v) solution of Bovine Serum Albumin (BSA) in PBST for 1 hour at room temperature on an orbital shaker. Grains were briefly rinsed in PBST (PBS, 0.1% Tween-20) and then incubated with mouse monoclonal antibodies in PBST for 1h at room temperature with agitation. The antibodies used and their dilutions were as follows: i) 1:100 IFRN 0610 mouse monoclonal antibody which recognizes an epitope (QQSF) common to many gliadins and to LMW-GS but not to HMW-GS; ii) 1:100 S3B512 mouse monoclonal antibody, raised against a peptide from the γ-gliadin repetitive domain (PEQPFPQGC) specific for γ-gliadins (INRA, Nantes, France).

Grains were washed in PBST and then incubated for 1 hour with an anti-mouse IgG gold conjugate (2 nm) diluted 1:50 from goat, used at 1:5000 dilution. After rinses in PBST and distilled water, the gold signal was silver-enhanced using the Aurion silver enhancement kit for 30 min at 22°C. After repeated rinsing with distilled water, the samples were dried in a desiccator. Dried samples were coated with carbon, using a carbon rod evaporator. The grains were then imaged in the JEOL JSM-6360 LV using a dry stage setting.

2.4 Sample preparation for immunofluorescence and light microscopy

Transverse sections (approx. 1 mm thick) were cut from the middle of each seed. Sections were fixed for 8 h at room temperature in 2.5 % (w/v) paraformaldehyde and 0.5 % (w/v) glutaraldehyde in 0·1 M Sorenson's phosphate buffer (prepared with $NaH_2PO_4.2H_2O$ and $Na_2HPO_4.12H_2O$), pH 7.2. After three rinses in buffer, the specimens were dehydrated in an ethanol series, infiltrated with LR White Resin (medium grade, TAAB L012) for several days and polymerized at 55 °C.

Semi-thin (1 μm) sections were cut using a Reichert-Jung Ultracut ultramicrotome, collected on drops of distilled water on multi-well slides coated with poly-L-lysine hydrobromide (Sigma P1399) and dried on a hot plate at 40 °C. Sections for general morphology and observation of protein bodies were stained with 0·01 % (w/v) Toluidine Blue in 1 % (w/v) sodium tetraborate, pH 9, and examined with bright-field optics on a Zeiss Axiophot microscope. Three independent batches (each batch was a seed from a different variety) were performed in this assay; for each seed, three different sections were analysed.

2.4.1 Immunofluorescence

Fixed and resin-embedded grain sections were briefly rinsed with PBST [PBS, Sigma A4417, 0·1 % (v/v) Tween 20, pH 7·4] and incubated in blocking solution [5 % (w/v) BSA (Sigma A7638) diluted in PBST] for 40 min at room temperature. This step was followed by incubation in the primary antibodies diluted in 1 % BSA (w/v) in PBST for 2 h at room temperature. Mouse monoclonal anti-IFRN 0610 (1:100), and mouse monoclonal anti-γ-gliadin (1:100) were individually tested. The unbound primary antibodies were removed by several rinses with PBST for a period of 20 min. The sections were then incubated for 1 h at room temperature in the dark with the secondary antibodies (Alexa Fluor 568 goat anti-mouse IgG; Invitrogen A-11001, A-11004) diluted 1:250 in 1% BSA in PBST. Finally, the slides were rinsed twice with PBST and three times with PBS.

Sections were examined with a Zeiss Axiophot epifluorescence microscope. A Retiga Exi CCD digital camera (QImaging, Surrey, BC, Canada) and MetaMorph software version 7·5·5 (Molecular Devices, Sunnyvale, CA, USA) were used to acquire the images.

2.4.2 Colorimetric assay for light microscopy

Grain sections collected on poly-lysine coated slides were pre-incubated for 30 min in a blocking solution made up of 0.3% Tween20 (Sigma-Aldrich) and 3% BSA (Sigma-Aldrich) in phosphate-buffered saline (PBS) (Sigma-Aldrich) solution at pH 7.4. Samples were then incubated for 2 h in primary R5-HRP conjugated monoclonal antibody, and diluted 1:100 in antibody buffer (1% BSA, 0.05% Tween 20 (Sigma-Aldrich) PBS solution). After three washes in a 0.3% Tween 20 PBS solution, samples were incubated in 1-Step™ Ultra TMB-Blotting revelation solution for 2 minutes

and then analyzed with bright-field optics on a Zeiss Axiophot microscope. Three independent batches (each batch was a seed from a different variety) were performed in the analysis; for each seed, three different sections were analysed.

2.5 Image Processing assay

Digital Image Processing and Analysis ImageJ, an open source java program, was used to quantify the microscopy images. Prior to analysis, three sections of the images from each sample in immunofluorescence and colorimetric experiments, were converted to 8-bit grayscale. A global thresholding was used for each image and the average intensity MGVs (Mean Grey Values) were calculated using the ImageJ program.

2.6 Statistical analysis

Statistics were performed in immunogold, colorimetric and immunofluorescence experiments.

For immunogold analysis, data are reported as the means of five replicates. For each seed, three different area were analysed. Mean differences were compared by unpaired Student's t-test. A p-value < 0.01 was considered significant. For colorimetric and immunofluorescence experiments, three grains from each treatment (CG and GFG) were considered; for each seed, three different sections were analysed. These data were used to run a Monte Carlo simulation and build a statistical population with at least 300 data points.

The results from antibody assays were preliminarily tested to verify the normal distribution of the population and then analysed through a multifactorial ANOVA using treatment (control or gluten-friendly) and variety (variety 1, variety 2 or variety 3) as categorical predictors. Tukey's test with $P<0.05$ was used as the *post-hoc* comparison test to pinpoint the significant differences. The effect of each predictor was reported as a "graph of hypothesis decomposition". Paired comparison was performed through a t-test ($P<0.05$).

The results from image processing were converted into an arbitrary scale from 0 to 4 to assess the presence of protein body type 1 (CG) or type 2 (GFG). These results were analysed through the Friedman's nonparametric test ($P<0.05$) and reported as median, minimum and maximum values, 1st and 3rd quartiles of the statistical population. Finally, control and gluten-friendly image processing data were analysed through a Principal Component Analysis. Statistics were performed using the Statistica for Windows software ver. 12.0 (Statsoft, Tulsa, OK, USA).

3. Results and discussion

3.1 Effect of Gluten-Friendly™ technology on wheat kernel endosperm morphology

Given that Gluten-Friendly™ technology induced significant changes in gluten proteins (Lamacchia et al., 2016), endosperm structure was analyzed by SEM (Scanning Electron Microscopy) to better understand the effect of this technology. SEM provides unique three-dimensional views of membranes and cytoplasm, otherwise not available with thin-sectioned endosperm. Several samples of CG (control grains) and GFG (gluten-friendly grains) were cleaved transversally into two halves and prepared for CPD (Critical-Point Drying) and freeze-fracturing analysis, and SEM-analyzed at a high voltage of 20.0 kV. CPD preserves the structure of cells with minimum alteration from the natural state with regard to volume, morphology and spatial relationships of organelles and macromolecules, minimum loss of tissue contents, and protection for samples against subsequent treatments including rinsing, dehydration, vacuum and exposure to the electron beam (Hayat, 1989). Fig. 1 shows representative micrographs of CG and GFG with textural patterns of the pericarp, aleurone, subaleurone and starchy endosperm in the mature grain. GFGs did not show differences from CGs and were very well preserved after microwave treatment.

The aleurone layer is the outermost cell layer of the endosperm tissue; its cells appear square or rectangular and are characterized by thick cell walls. Subaleurone cells, which are peripheral endosperm cells, constitute one cell layer, made up of cells that are smaller than the subsequent inner endosperm cells. The endosperm cells below the subaleurone cells are prismatic, while central cells are more variable in shape. Fig. 1 pinpoints the differentiation of starch granules into two main size categories: type A (large granules) which are typically 10-25 μm in size, and type B (small granules), ranging between 2-10 μm. Starch granules did not differ in number and shape in GFG and CG samples; moreover, they were dispersed into the continuous protein matrix (mainly proteins and residual cytoplasmic constituents).

The profiles of the freeze-fractured cleaved seeds were also studied, as shown in Fig. 2. Freeze-fracturing provided a technique to study the morphology of endosperm tissue in GFG samples, without many of the problems associated with fixed tissues. In fact, structural features may represent artefacts in chemically-fixed material (Bechtel & Barnett 1986; Bechtel 1983; Miflin, Field & Shewry, 1983). Currently, the most effective alternative to chemical fixation is cryofixation. All freezing methods have the common aim of preserving the sample in its native/original state without artefacts. Samples can be visualized in their native hydrated state without pre-treatment, preserving the natural morphology of cells and tissues. This effect is achieved by removing heat at such a rapid rate that water molecules form amorphous vitreous ice, thus avoiding the destructive effects of crystalline ice on cellular ultrastructure. When rapid freezing attains the vitrification temperature, viscosity reaches a level which prevents movement, thus immobilizing all the molecules in a cell within milliseconds (Moor, 1987).

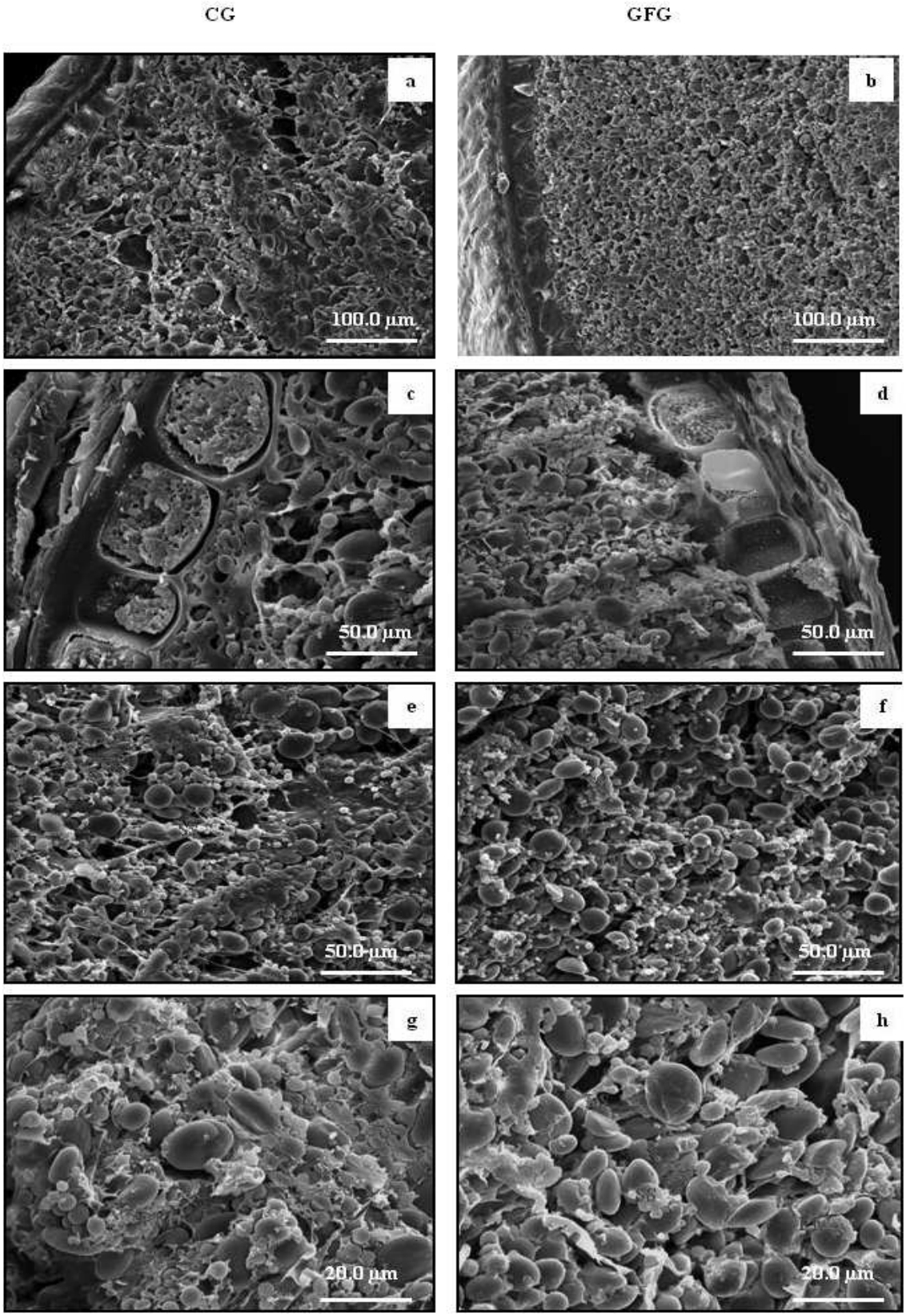


Fig. 1. SEM of transversally-cleaved CPD-treated control grain (CG) and Gluten-Friendly Grain (GFG) samples, (a, b) showing the pericarp and aleurone, subaleurone and endosperm cells x50; (c, d) Aleurone cells (Al) and the outermost endosperm cells (subaleurone layer) (x500); (e, f) Large starch granules (LS) and small starch granules (SS) as well as protein matrix (PM) in endosperm tissue (x1000); (g, h) A higher magnification of large and small starch granules and protein matrix (x2000).

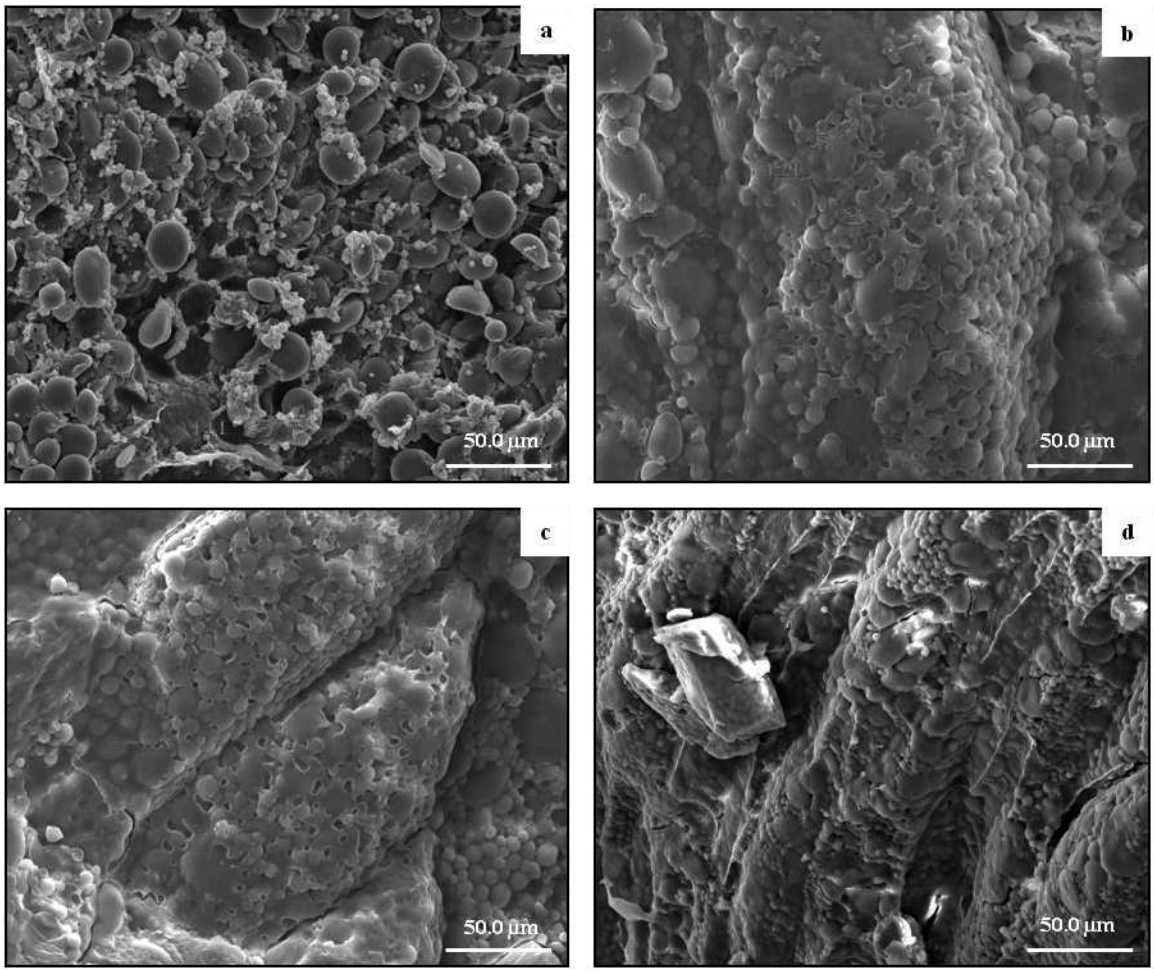


Fig. 2. SEM of transversally-cleaved freeze-fractured control grain (CG) and Gluten Friendly Grain (GFG) samples; (a) CG subaleurone cells rich in protein matrix (1500x); (b)(d), GFG subaleurone cells completely embedded into a confluent protein matrix (1500x). Bars in grain samples correspond to 50.0 µm.

Thus a freeze-fracturing technique was used and replicas of unfixed wheat endosperm revealed a different pattern of protein matrix in endosperm tissue. Specifically, the results showed (Fig. 2) a different protein matrix deposition pattern in GFG compared to CG samples.

Micrographs of GFG clearly demonstrate the presence of a thick, confluent protein matrix in prismatic endosperm cells, with complete embedding of the starch granules. These results are in accordance with previous works that suggest that under heat stress, the endosperm of the kernels might appear increasingly aggregated, with the starch granules embedded in the protein matrix and a dense cellular structure (Pyler, 1988, Dias & Lidon, 2009). The different results obtained with CPD and freeze-fracturing may be explained by the fact that CPD treatment is more invasive, exposing samples to chemical reagents and dehydrating forces, that could break the interactions among aggregated proteins.

3.2 Effect of Gluten-Friendly™ technology on gluten protein structure

Gluten contains hundreds of proteins, which are present either as monomers or as oligomers/polymers, linked by inter-chain disulphide bonds (Wieser, 2007; Shewry & Halford, 2002) and characterized by high levels of glutamine and proline (namely prolamin) residues, clustered in either polyglutamine sequences or in repeating glutamine/proline sequences. The effects of Gluten-Friendly™ technology on gluten protein structure was evaluated by *SEM–Immunogold* and *Immunofluorescence* experiments with two monoclonal antibodies specific for repeated sequences common to gliadin and LMW fractions. We used the IFRN 0610 monoclonal antibody, that recognizes epitopes QQSF, QQSY which are common to many gliadins and LMW-GS, but not HMW-GS, and the mouse γ-gliadin monoclonal antibody, that recognizes the repetitive domain PEQPFPQGC present in the γ-gliadin fraction. The protocol was carried out on five CG and GFG samples to evaluate the differences in the amount of specific labelling.

Fig. 3 shows the results from *SEM-Immunogold* analysis. Fig. 3A shows CG and GFG samples after specific labelling with the two antibodies. Energy Dispersive Spectroscopy (EDS) confirmed that it was silver-enhanced gold labelling (and not an artefact). This technique works by detecting an alternative signal given off by the sample (X-rays) rather than those usually detected for producing high-quality images (secondary electrons). The X-rays emitted by a sample after irradiation with the electron beam are unique to each element, and therefore elemental identification is possible by detecting these X-rays' energy and comparing them to known standards. A typical EDS trace is shown in Figure 3B. The sections labelled in Fig. 3A indicate a point on the surface of the endosperm which was chosen to be analyzed with EDS. The trace in the same figure shows the elements present in the labelled area (65x51nm). This trace shows many elements are present, including carbon, oxygen and silver, confirming that the flecks previously assumed to be silver-enhanced gold labelling were indeed so. EDS was used for each sample shown in the following result to confirm the suspected labelling. Fig. 3A shows that labelling can be observed within the protein matrix situated between the starch granules, but not on the starch granule surface itself, nor anywhere else in the grain, indicating that specificity has been maintained. Labelling present on the protein matrix of CG samples could be defined as "heavy" compared to GFG samples. The results are summarized in Fig. 3C and show a significant ($p<0.01$) decrease in silver concentration in GFG seeds compared with CG, after labelling with the two antibodies. The decrease was about 89% for monoclonal antibody 0610, and 87% for the γ-gliadin antibody, respectively. Fig. 4 A shows the results from *Immunofluorescence* experiments. In GFG samples, a strong and significant decrease in signal intensity was observed after labelling with antibody 0610 and the antibody specific for γ-gliadin compared to the CG samples, in agreement with the *SEM* results.

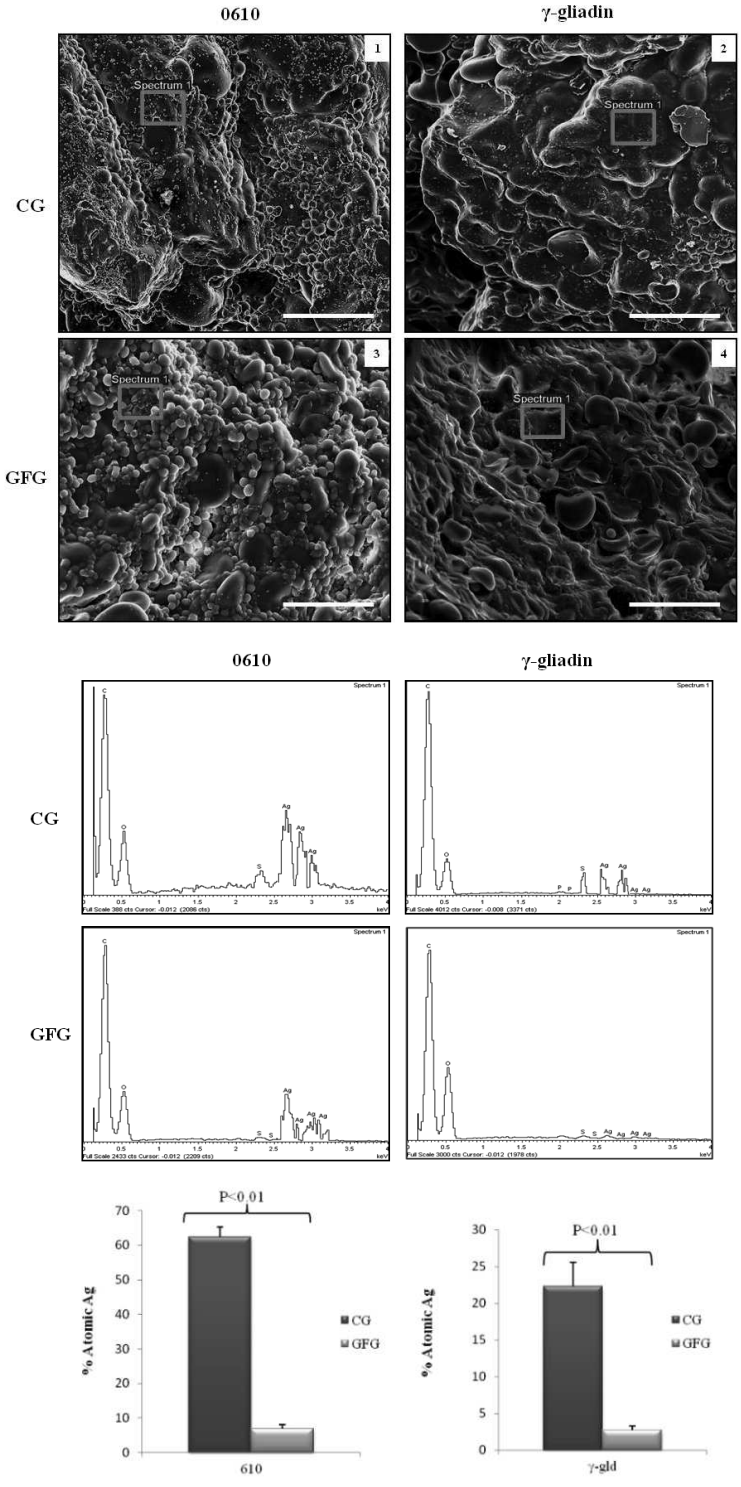


Fig. 3. (A) SEM-Immunogold labelling in control grain (CG) and Gluten Friendly Grain (GFG) samples with corresponding Energy Dispersive Spectroscopy (EDS) trace. (1) CG subaleurone immunogold labelled with IFRN 0610 antibody; (2) CG subaleurone immunogold labelled with c-gliadin antibody; (3) GFG subaleurone immunogold-labelled with IFRN 0610 antibody; (4) GFG subaleurone immunogold labelled with c-gliadin antibody. Bars in grain samples correspond to 50 lm. (B) Energy Dispersive Spectroscopy (EDS) trace corresponding to SEM-Immunogold labelling in CG and GFG samples. (C) Histogram summarizing the atomic silver % (mean ± SD) detected with Energy Dispersive Spectroscopy (EDS) in CG and GFG samples after the labelling with 0610 and c-gliadin antibodies. A p-value < 0.01 was considered significant.

Labelling was measured by quantifying the light signal and correlating it to a greyscale, through the ImageJ software. Data reported in Fig.4B expressed as MGVs (mean grey values) summarize this reduction: 91.71% ($p < 0.001$) and 90.61% ($p < 0.001$) for 0610 and γ-gliadin antibodies, respectively. Fig 4B shows the hypothesis decomposition trend and, for each treatment, three seeds from three different varieties are reported (sample 1, sample 2, sample 3). Only the predictor "treatment" (control or gluten-friendly) played a significant role in the decrease in MGV, whereas the effect of variety was not significant.

These results confirm that Gluten-Friendly™ technology induces significant changes in gluten proteins, thus reducing cross-reactivity with antibodies that recognize almost the entire range of gluten proteins through the sequences QQSF, QQSY, PEQPFPQGC. Lamacchia et al. (2016) suggested that the brief high temperatures generated by microwaves applied to the hydrated caryopses lead to conformational and/or structural changes in the proteins, as evidenced by the increase in total cysteine levels. Furthermore, the analysis by SDS-PAGE of protein fractions under reducing and non-reducing conditions showed that microwave treatment causes neither a decrease in the number of the bands nor a decrease in their intensity (Lamacchia et al., 2016); thus, a rearrangement of the secondary and/or tertiary structure in gluten proteins with a different spatial conformation of the aminoacid sequences could be suggested. Enlargement of part of the micrographs (Fig. 5) highlighted another interesting feature, i.e. a strong merging of protein bodies in the CG and GFG samples. Gluten proteins comprise the major grain storage proteins, which are deposited in protein bodies in the developing starchy endosperm. The individual protein bodies range in diameter up to about 20 μm. However, as the endosperm cells fill with starch, the protein bodies should become disrupted and finally coalesce to form a matrix of storage proteins surrounding the starch granules in the mature dry tissue (Shewry, Tatham, Barro, Barcelo, Lazzeri, 1995). Indeed, micrographs of the samples revealed that the coalescence of protein bodies in mature wheat grains was complete and resulted in a continuous proteinaceous matrix surrounding the starch granules.

Nevertheless, it was possible to still distinguish protein bodies (Fig. 5 A) and highlight differences in the merging level of protein bodies in GFG with respect to CG samples (Fig. 5 A). In the GFG samples, protein bodies (PB-type2) were completely merged in the protein matrix, which appeared more homogeneous and confluent, showing a higher level of aggregation when compared to CG samples (PB-type1). Data were collected on four semi-thin sections (1μm) of six different CG and GFG samples. The differences were pointed out through Friedman's nonparametric test, because a preliminary statistic revealed that the scores in the arbitrary scale did not follow a normal trend. The Friedman test was run on the median value, as reported in Fig 5B and highlighted a strong increase in PB-type 2 in GFG. This result was also confirmed by using variety as an input value, thus

suggesting that technology and not variety plays a fundamental role in protein aggregation (data not shown). A multivariate approach (PCA) confirmed that PB-type 1 and PB-type 2 were negatively related.

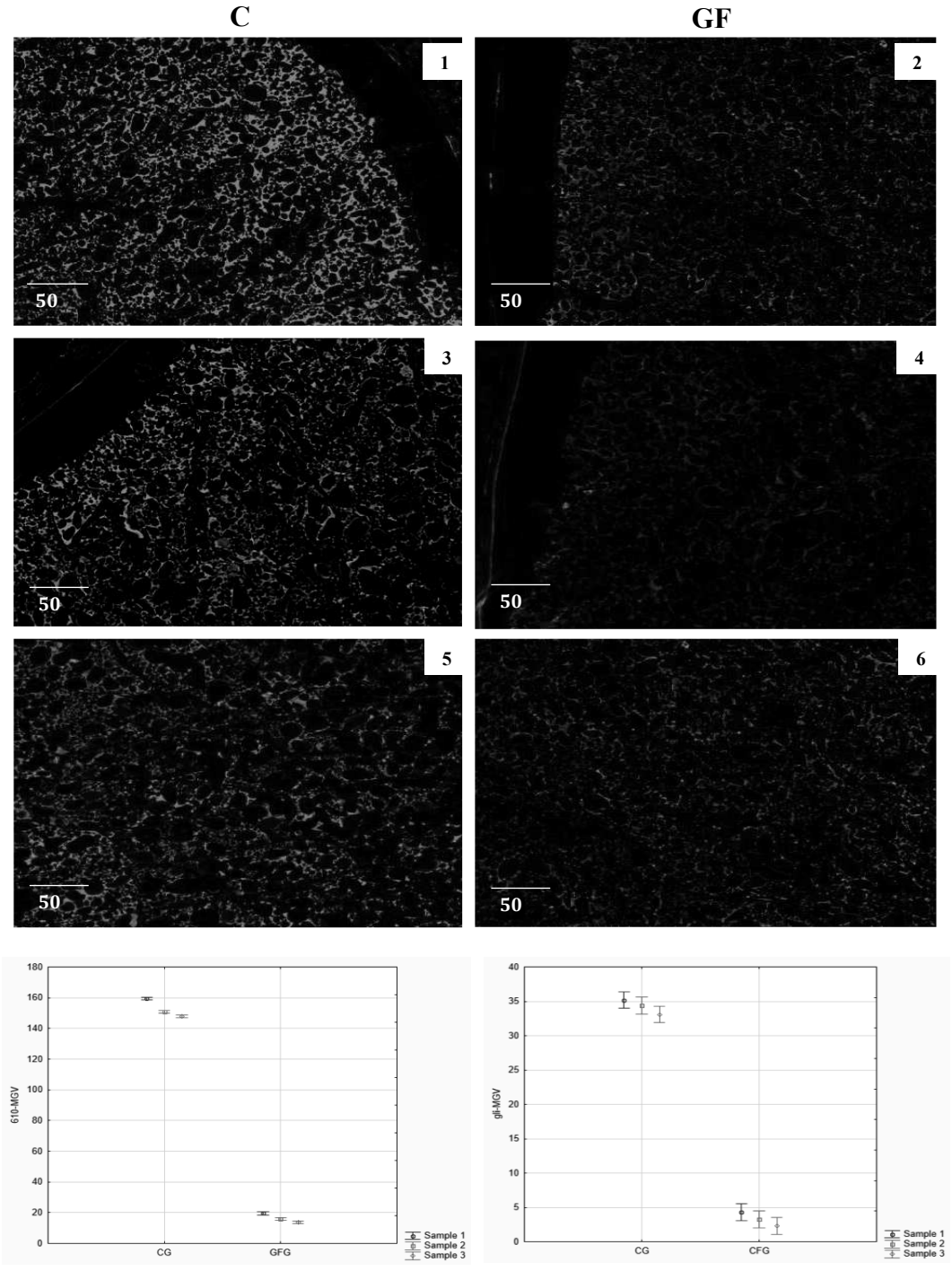


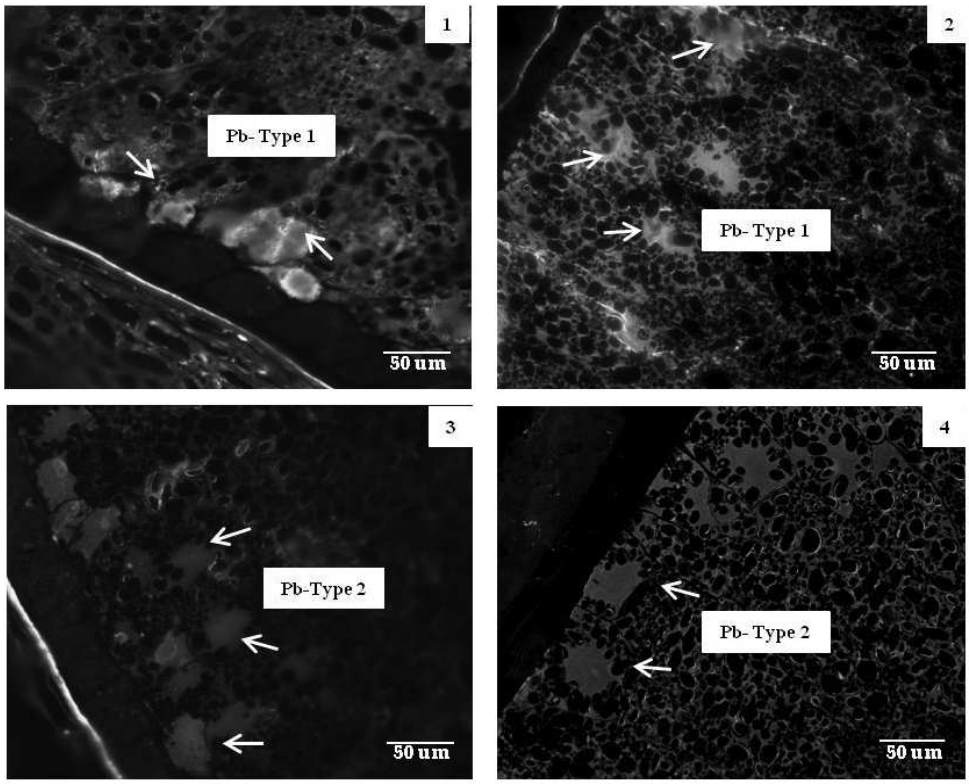
Fig. 4. (A) Micrographs of control grain (CG) and Gluten Friendly Grain (GFG) semi-thin slices immunolabelled with 0610 and c-gliadin monoclonal antibody in immunofluorescence experiments. (1) CG subaleurone immunolabelled with IFRN 0610 antibody; (2) GFG subaleurone immunolabelled with IFRN 0610 antibody; (3) CG subaleurone immunolabelled with c-gliadin antibody; (4) GFG subaleurone immunolabelled with c-gliadin antibody; (5) CG starchy endosperm immunolabelled with IFRN 0610 antibody; (6) GFG starchy endosperm immunolabelled with IFRN 0610 antibody. (B) Two-way ANOVA: Hypothesis decomposition for the interaction kind of sample (1, 2, or 3) and treatment (CG and GFG samples) on the mean grey value (MGV). Bars denote 95%-confidence intervals.

These results suggest that aggregation may be the primary cause for the reduced cross-reactivity of proteins, rather than a change in secondary and/or tertiary structure. In any event, these findings are in accordance with analyses performed with SEM using freeze-fracturing techniques and with the fact that gluten proteins are susceptible to heat treatment and polymerize during heating (Schofield, Bottomley, Timms, & Booth, 1983). Gluten protein behaviour when subjected to relatively high temperatures has been studied by a number of workers and indeed the incorporation of gliadin monomers in the glutenin network through covalent bonds has been highlighted (Singh & MacRitchie, 2004; Redl, Morel, Bonicel, Vergnes, & Guilbert, 1999). However, Lamacchia, Baiano, Lamparelli, La Notte & Di Luccia (2010) showed, for the first time, that the application of high temperature to wheat grains generated protein polymerization in which gliadins did not cross-link with glutenins. The explanation for this phenomenon was that, within the kernel, the gluten protein network has not yet formed and gluten proteins are deposited in different protein bodies. Furthermore, Lamacchia et al., (2016) confirmed these results, showing that the application of a short burst of high temperature to mature hydrated wheat kernels using microwave energy leads to protein polymerization between proteins of the same class (i.e. gliadin-gliadin, glutenin-glutenin, albumin-albumin) and only through disulphide bonds (evidenced by the SE-HPLC chart and SDS-PAGE analysis). However, this does not seem to be in accordance with the findings from the SEM and immunofluorescent experiments where images show a strong aggregation among all wheat kernel proteins. One explanation for this could be that the strong aggregation between different classes of proteins in seeds is due to ionic and/or hydrophobic interactions, caused by a rearrangement of the secondary and/or tertiary structure of the gluten protein molecules during heating.
Such aggregation was not visible through SE-HPLC and SDS-PAGE (Lamacchia et al., 2016) because of the use of sodium dodecyl sulphate-denaturing conditions in the extraction buffer.

3.3 Effect of Gluten-Friendly™ technology on gluten protein antigenicity

The short sequence of amino acids that binds with the antibody to elicit immune reaction is called the antigenic epitope. The most common antigenic epitopes of gluten proteins are HLA DQ2 (Human Leukocyte Antigen), characterized by multiple proline (Pro) and Gln residues (Kim, Quarsten, Bergseng, Khosla, & Sollid, 2004). Tanabe (2008) also reported Gln-Gln-Gln-Pro-Pro as the major motif of wheat gluten protein to act as an antigenic epitope.
Currently thirty-one aminoacid peptide sequences in the prolamins of wheat and related species have been defined as being coeliac-toxic: these are often referred to as “coeliac epitopes”. However,

mapping is incomplete and the number of distinct epitopes is a matter of ongoing discussion (Sollid, Qiao, Anderson, Gianfrani, Konig, 2012).



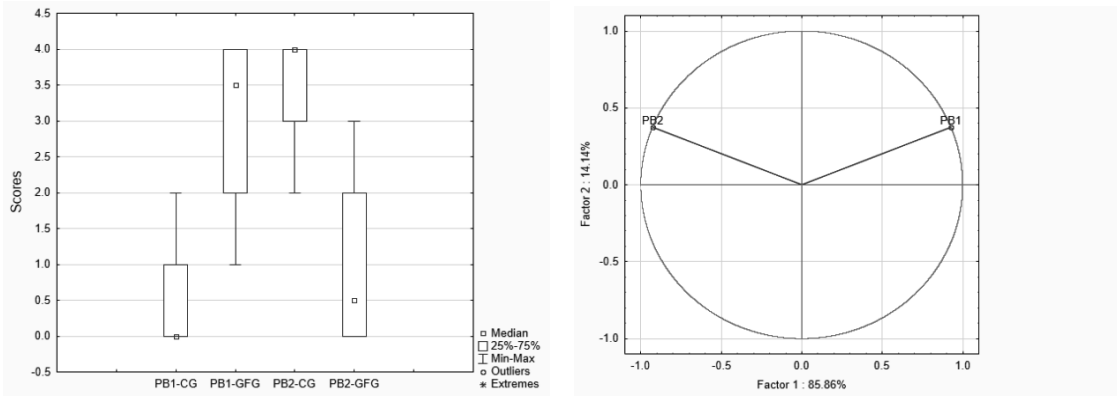


Fig. 5. (A) Enlargement of part of micrographs of control grain (CG) and Gluten-Friendly Grain (GFG) semi-thin slices immunolabelled with 0610 and c-gliadin monoclonal antibody in immunofluorescence experiments showing a strong merge of protein bodies in CG and GFG samples, respectively. (1, 2) Protein bodies type 1 (PB-type1) from CG samples; (3, 4) Protein bodies type 2 (PB- type2) from GFG samples. (B) Box-Whisker plots on the protein bodies. The differences were identified through Friedman's nonparametric test, which highlighted significant differences between CG and GFG samples for both PB-type1 and PB-type 2. PB-type1 and PB-type 2 showed discontinuous scores, ranging from 0 to 5.

These epitopes are located in the repetitive domains of the prolamins, which are proline- and glutamine-rich, and the high levels of proline in their sequences may reduce their susceptibility to protease activity in the GI tract. The prolamin-reactive T-cells (T-lymphocytes) of CD patients also recognize these epitopes to a greater extent when specific glutamine residues in their sequences have been deamidated to glutamic acid by a tissue transglutaminase (tTG2). This binding enables the formation of a stable peptide-MHC complex, which is important in the anti-prolamin T-cell response (Sollid et al., 2012).

The effect of Gluten-Friendly™ technology on gluten protein antigenicity was evaluated by *Light Microscopy* using the R5 monoclonal antibody which recognizes the most repetitive potential coeliac-toxic pentapeptide epitope, glutamine-glutamine-proline-phenylalanine-proline (QQPFP) in gluten proteins and the epitopes LQPFP, QLPYP and PQPFP in the sequence of the recently-described potent inducer of gut-derived human T-cell lines in coeliac patients, the A gliadin 33-mer peptide of residues 57–89 (LQ LQPFPQPQLPYPQPQLPYPQPQL–PYPQPQPFP) (Arentz-Hansen, Korner, Molberg, Quarsten, Vader, Kooy, 2000). Light microscopy was chosen because the R5 monoclonal antibody was provided horseradish peroxidase-conjugated. A colorimetric assay was developed, where the antibody was revealed with a specific enhanced single-component horseradish peroxidase (HRP) substrate for immunohistochemistry. The revealing solution, which contains soluble TMB (3,3',5,5'-tetramethylbenzidine), reacts very quickly with horseradish peroxidase enzyme to produce an insoluble dark blue precipitate.

Results are shown in Fig. 6A, where it was possible to detect a decrease in the signal emitted in GFG samples, compared to CG, both in the subaleurone and the crease portion of the seeds. The colour intensity was then measured through the ImageJ software. Data reported in Fig. 6B, expressed as mean grey values, summarize this reduction: 89.19% ($p<0.001$) for the subaleurone layer and 81.6% ($p<0.001$) for the crease tissue.

The reduced gluten antigenicity in GFG samples observed with the R5 colorimetric assay was in accordance with the *SEM–Immunogold* and *Immunofluorescence* experiments and with Lamacchia et al. (2016) that showed a drastic reduction (99%) in the levels of detectable proteins, treated with Gluten-Friendly™ technology, tested for their ability to bind the specific monoclonal antibody R5 in a sandwich Elisa test. R5-Elisa includes an extraction procedure allowing aggregates of gliadins generated by the heat process to be solubilized (Valdés, García, Llorente, & Méndez, 2003). On this basis, a rearrangement of the secondary and tertiary structure in gluten proteins, with a different spatial conformation of the toxic sequences, induced by Gluten-Friendly™ technology, and not a strong aggregation among proteins, can explain the significant reduction in antigenicity in GFG samples and therefore a significant reduction in cross-reactivity of gluten proteins with their own

antibodies. This is well supported by the fact that the availability of antigenic fragments in gluten depends on the secondary and tertiary structure of the protein as well as on the SS bonds that stabilize particular conformations of epitopes to bind with the antibody (Waga, 2004).

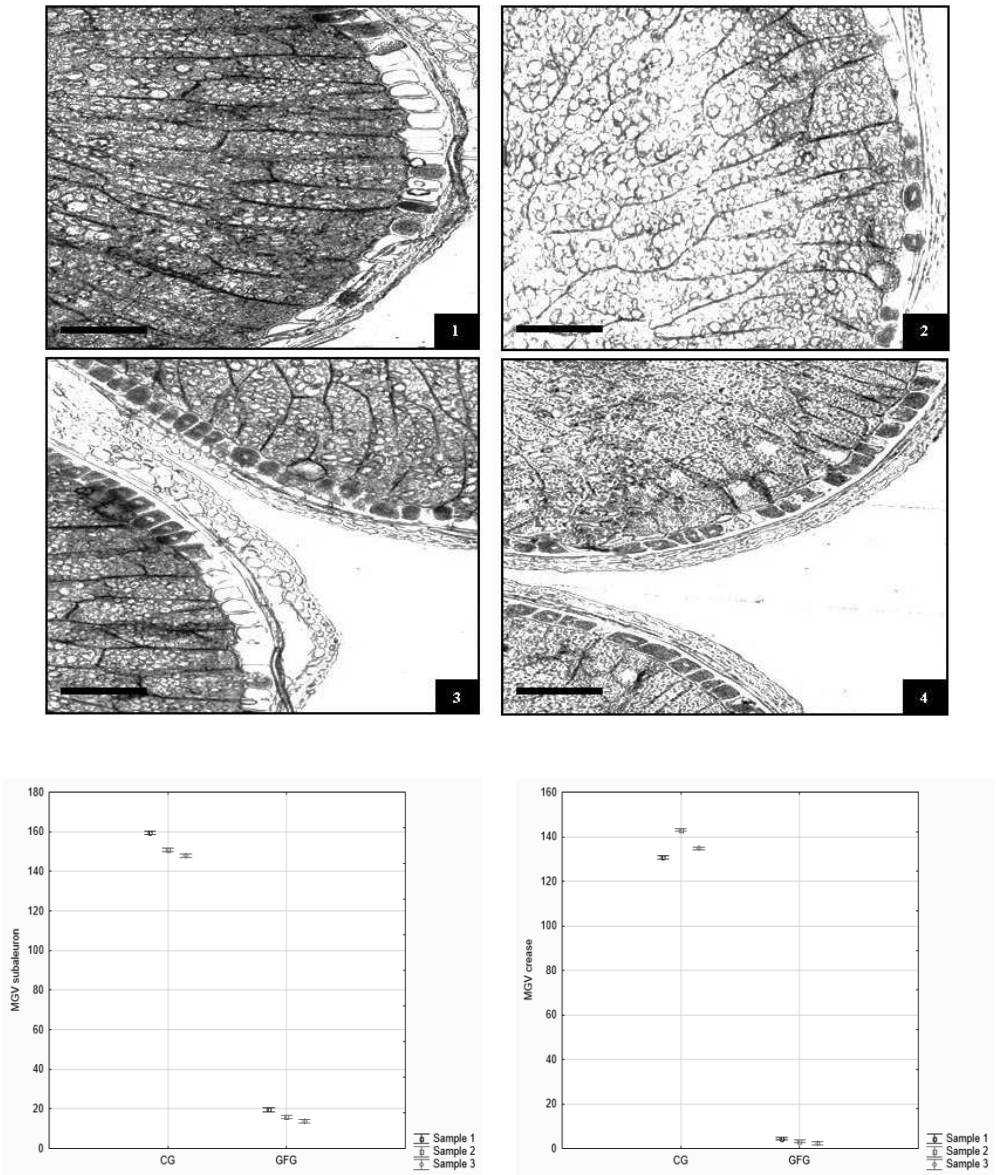


Fig. 6. (A) Micrographs of control grain (CG) and Gluten Friendly Grain (GFG) semi-thin slices immunolabelled with R5 monoclonal antibody. (1) CG subaleurone layer; (2) GFG subaleurone layer; (3) CG crease portion; (4) GFG crease portion. Bars in cross-sections correspond to 100 µm. (B) Two-way ANOVA: Hypothesis decomposition for the interaction kind of sample (1, 2, or 3) and treatment (CG and GFG samples) on the mean grey value (MGV). Bars denote 95%-confidence intervals.

The reduced gluten antigenicity observed in this study also confirms preliminary results showing that flours from grains treated with Gluten-Friendly technology did not induce production of the inflammatory cytokine, interferon gamma, when tested, after digestion and deamidation, on gut-derived human T-cell lines of coeliac patients highly reactive to 33-mer, omega and gamma peptides (Lamacchia et al., 2015).

4.Conclusion

Gluten-Friendly™ technology induced a significant aggregation of proteins in seeds and deep changes to the gluten protein structure. Protein bodies were completely merged in the protein matrix, which appeared more homogeneous and confluent when compared with control samples. The cross-reactivity of gluten proteins with antibodies that recognize both the almost entire range of gluten proteins and antigenic epitopes was significantly reduced. The present study confirms the results from our previous work which showed, for the first time, the mechanism by which a chemical-physical treatment abolishes the antigenic capacity of gluten. Furthermore, our data suggest that chemical changes are caused by a rearrangement of the secondary and tertiary structure, involving a different spatial conformation of the sequences, also of the so-called antigenic ones. These changes may allow a new kind of aggregation among different classes of wheat endosperm proteins, only through hydrophobic and/or ionic interactions.

Acknowledgement

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Chapter 4

Chemical, rheological and sensorial evaluation of Gluten-Friendly™ Flour

(In preparation)

ABSTRACT

The chemical, rheological and pasting porperties of Gluten Friendly Flours was investigated. The gluten became soluble in aqueous solution and it was not possible to isolate it using the Glutomatic apparatus. A decrease of dough elasticity was detected using an extensiograph, dough stability decreased from 5 to 2 min, whereas farinograph water absorption of Gluten Friendly Flour was higher (+10%) than control flours. Alveograph curve configuration ratio (P/L) increased from 0.63 to 6.31, whereas pasting properties were not significantly different from that of control flours.

Although deep modifications of gluten proteins, Gluten Friendly Flours was able to form dough and leaven and produce bread. The sensory qualities of Gluten Friendly Bread did not differ significantly ($p<0.05$) from the Control Bread.

1. Introduction

Gluten is one of the earliest protein fractions described by chemists, (a first description by Beccari was in 1728), and it is defined as the "cohesive, visco-elastic proteinaceous material" that remains when wheat dough is washed to remove starch granules and water soluble constituents (Shewry, 2009; Wieser, 2007). Gluten is formed by storage proteins necessary for plant germination (Shewry, 1999). In these respects, the wheat storage proteins may not differ much from those of other cereals (Shewry, 2009; Shewry & Halford 2002), however, the distinctive feature that makes wheat unique is, precisely, the visco-elasticity of gluten.

Gluten contains hundreds of proteins, which are present either as monomers or as oligo- and polymers, linked by inter-chain disulphide bonds (Wieser, 2007; Shewry & Halford, 2002), and characterized by high contents of glutamine and proline (namely prolamins), and by low contents of charged amino acids.

Traditionally, gluten proteins have been divided according to their solubility in alcohol-water solutions (e.g., 60% ethanol), as the soluble gliadins and the insoluble glutenins (Osborne, 1924). These properties are largely determined by the inter-chain disulphide bonds, with the glutenins consisting of disulphide-stabilized polymers. Reduction of these inter-chain bonds allows the separation of the glutenin subunits into low molecular weight (LMW) and high molecular weight (HMW) groups (Figure 2). In contrast, the alcohol-soluble gliadin fraction consists mainly of monomeric proteins, which either lack cysteine (ω-gliadins), or have only intra-chain disulphide bonds (α-type and γ-type gliadins). Both fractions are important contributors to the rheological properties of dough (Figure 2), though their functions are divergent. Hydrated gliadins have little elasticity and are less cohesive than glutenins, and contribute mainly to the viscosity and extensibility of the dough. In contrast, hydrated glutenins are both cohesive and elastic and are responsible for

dough strength and elasticity (Field et al., 1983; Payne, 1987). A proper mixture of both fractions is essential for the quality of the end product. However, of particular importance are the glutenin polymers, and it is well established that strong (i.e., highly visco-elastic) doughs contain high proportions of HMW glutenin polymers (Field et al., 1983). Numerous studies are consistent with the hypothesis that the HMW subunits form an elastomeric polymer network which provides a "backbone" for interactions with other glutenin subunits and with gliadins (Shewry et al, 2001; Lindsay & Skerrit, 1999). There is no doubt that this network is mainly stabilized by inter-chain disulphide bonds (Shewry & Tatham, 1997; Wieser, 2003). Additional covalent bonds formed during dough making are tyrosine-tyrosine and thiyl-tyrosine crosslinks between gluten proteins (Tilley et al., 2001, Lamacchia et al., 2011). Celiac disease is the most common food induced enteropathy in humans. CD is strongly associated with particular HLA genotypes, as only individuals carrying the DQA1*0501 and DQB1*0201 (DQ2), or DQA1*0301 and DQB1*0302 (DQ8) alleles develop the disease. In these subjects, the consumption of cereals containing gluten causes a chronic inflammatory process leading to lesions in the small intestine and a dysfunction in nutrient absorption (Shuppan et al., 2005). The CD treatment, therefore, is based on a strict gluten-free diet throughout the patient's lifetime. Though this dietary regimen guarantees the full recovery of small intestine architecture and functions, for many patients it is strongly restrictive, especially for social events and during travelling. In addition, it is a great task for the food industry to provide safe food that partly resembles in taste and appearance pasta, bread and other baked goods. An alternative way to exclude gluten is to detoxify it without affecting the technological performances of the resulting flour and dough. Recently, we have developed a new and innovative detoxification method of gluten proteins from cereal grains (Italian patented method n°: 0001414717, also filed under the Patent Cooperation Treaty, application no. PCT/IB2013/000797) (Lamacchia, Di Luccia, & Gianfrani, 2013, 2015a), aiming to combine the nutritional and technological properties of wheat proteins with safety for coeliac sufferers and other gluten-sensitive subjects. This innovation is usually referred to as ''Gluten FriendlyTM" and relies on 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 and induce a structural change in gluten proteins (Lamacchia, Landriscina, & D'Agnello, 2016). This modification abolishes the antigenic capacity of gluten (Lamacchia et al., 2016) and reduces the immunogenicity in vitro of the most common epitopes involved in coeliac disease (Lamacchia, Di Luccia, & Gianfrani, 2015b), without compromising the nutritional or the technological properties necessary to process semolina in pasta and flours in bread and other baked goods (Lamacchia et al., 2016). Moreover, Gluten FriendlyTM bread can also partly correct and positively modify the quali-quantitative composition of the microbiota of coeliac people in a model system (Bevilacqua, Costabile, et al., 2016). The

objectives of this work are to evaluate the chemical composition, rheological and bread making properties of Gluten Friendly Flours (GFF) and Control Flours (CF).

2. Materials and Methods

2.1 Raw Materials and microwave treatment

The wheat kernels (mixtures of soft wheat Canadian grains) used in this study were supplied by the Casillo group S.p.A. (Corato, Italy). Grains treated with microwaves were called Gluten-Friendly Grains (GFG) and were obtained by treating the caryopses, previously harvested and threshed, with microwave energy (Italian patented method n_: 0001414717 also filed under the Patent Cooperation Treaty, application no. PCT/IB2013/000797). The technology has since been 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 were dampened to 15–18% moisture; moisture was evaluated using a 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 was 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%.

2.2 Bread making

Bread was produced using the straight dough process, based on the following formulation: 100g of flour, 2,5% yeast, 2% salt, 5% sugar and variable absorption. Baking trials were carried out under laboratory conditions to optimize baking conditions. Dough was mixed to optimum consistency in a Kenwood mixer (Model A 907 D, Kenwood Ltd, England) with low speed of 85 rpm for 2 min. Final dough temperature was 30°C. The dough was fermented in a chamber maintained at 30 °C and 75 % relative humidity (RH) for 30 min, remixed, rounded, and again fermented for 25 min and
baked for 25 min at 220 °C. Baking was done at 220°C in an electric oven (Miwe Condo FP) until the golden brown colour was formed. The resulting bread samples were allowed to cool to room temperature (37°C) for 2 h before further analysis were carried out.

2.2 Chemical composition of flours

Protein, moisture and water absorption, were analyzed with official methods (AACC, 2000) using a Partens Inframatic analyzer (Model 9140, SE-126 53, Hägersten, Sweden). Dry gluten content and gluten index were analyzed using the Glutomatic 2200 system (AACCI Method 38-12.02). Alpha-amylase activity was determined with the Hagberg falling numbers instrument (Pertens model n° 1500, USA), based on (AACC, 2000) approved method.

2.4 Determination of the rheological properties of Gluten Friendly Flours

Farinograph and extensograph characteristics were determined according to the AACC 54-21 and AACC 54-10 methods, respectively (AACC, 2000). The following parameters were determined in a Brabender farinograph: water absorption percentage of water required to yield dough consistency of 500 BU (Brabender Units), dough development time (DDT, time to reach maximum consistency), stability (time during dough consistency is at 500 BU), mixing tolerance index (MTI, consistency difference between height at peak and to that 5 min later) and elasticity (band width of the curve at the maximum consistency). Brabender extensograph gave the resistance and maximum resistance to constant deformation (BU), energy (cm^2) and extensibility (E) (AACC 2000). Alveograph test was performed using an alveograph (Chopin (NG), France) following the AACC method (54-30A) (AACC, 2000). The following alveograph parameters were automatically recorded by a computer software program: tenacity or resistance to extension (P), dough extensibility (L), curve configuration ratio (P/L ratio), the deformation energy (W). The pasting properties of the flours were studied using a Brabender Amylograph (D-47055, Brabender Ohgduisburg, Germany) (method 22–12, AACC, 2000).

2.5 Bread crumb cell analysis

Crumb cell analysis were performed by scanning longitudinal and cross section of bread sample, 10 mm thick, on flatbed scanner (HP Scanjet 4400c). Images were taken from the center of the slice, and were analyzed by ImageJ software (National Institutes of Health, USA) according to Gonzales-Barron and Butler (2006). The crumb cell features chosen were the total number of cells, average cell per mm^2, average diameter per mm^2 and circularity. The characterization of the crumb structure was performed in triplicate.

2.6 Sensory evaluation

Loaves were cooled for 1-2 h at room temperature (25°C). Sensory evaluation was performed using 30 panelists, comprising staff members of the Casillo Group S.p.A., (Corato). Samples were randomly assigned to each panelist. The panelists were asked to evaluate each loaf for appearance, crumb texture and stability, crust and crumb colour, taste, aroma and overall acceptability. A 9-point hedonic scale was used where 1 = dislike extremely to 9 = like extremely.

2.7 Statistical analysis

All determinations were performed in triplicate. Analysis of variance (ANOVA) and Tukey's test (significance level $p \leq 0.05$) were used to determine the significance of the data. All statistical analyses and graphical representations were performed in STATISTICA 7.0®.

3. Results and discussion

3.1 Chemical composition of Gluten Friendly Flours

Gluten Friendly Flours (GFF), were analyzed chemically and the results are given in Table 1. The wheat flour moisture analysis results ranged from 12.1 for CF to 13.2 for GFF. Control flour and Gluten Friendly flours were analyzed for dry gluten and gluten index. The dry gluten content was decreased for GFF when compared to CF. The decrease was about 90% and was statistically significant ($p<0.05$). Furthermore, results showed that isolation of gluten from GFF was not possible and the gluten index for GFF samples was 0. These results suggest that microwave treatment induces significant modification of gluten proteins such that they become soluble in aqueous solution. (Lamacchia et al., 2016). The main reasons for the water-insoluble characteristics of gluten are the hydrophobic nature of its proteins, consisting of a large percentage of uncharged amino acid residues (glutamines Gln; asparagines Asn) and the presence of hydrogen bonds. By transforming the amides of Gln and Asn into carboxyl groups through deamidation, the solubility of proteins in wheat gluten is transformed with effects such as changing of the charge density, increasing the electrostatic repulsion and breaking the hydrogen bonds (Liao et al., 2010; Riha, Izzo, Zhang, & Ho, 1996). Alpha amylase activity (Falling number), ranged from 345 for GFF to 350 for Control flours (Table 1) and did not differ significantly ($p<0.05$). Falling number is a method aimed at determining the sprout damage and α-amylase activity in wheat grains which determines the flour quality for bread making (Wang et al. 2008). Amylases in doughs provide fermentable sugar for yeast and increase gassing power in doughs that are deficient in sugar.

Samples	Moisture (%)	Protein (%)	Dry Gluten (%)	Gluten Index	Alpha amylase activity (Falling No)
CF	12,1 a	12,03 a	7,5 a	80	345 a
GFF	13,2 b	12,01 a	1 b	NA	350 a

a, b= $p<0.05$

Table 1. Chemical characterization of Control Flours (CF) and Gluten Friendly Flours (GFF). Mean values in the same column followed by a different letter are significantly different ($p\leq0.05$).

3.2 Rheological properties of the dough

The alveographic characteristics of Gluten Friendly Flours (GFF) and Control flours (CF) are shown in Fig.1 and Table 2(A). The peak height (P) indicated the resistance that the dough offered to deformation and it is related to the tensile strength or stability that the dough exhibited during the

proofing stage of bread making (Pyler 1988; Mepba et al., 2007). The P values ranged from 52 mm for CF to 164 mm for GFF. The L values, which is a measure of dough extensibility, was significantly lower for treated flour as compared to control flours. The values for curve configuration ratio (P/L), indicating the ratio of elasticity and extensibility of the dough were significantly higher ($p<0.05$) for the treated flour than the control flours. The curve configuration ratio (P/L) is an index of gluten behavior. High values of curve configuration ratio may be indicated strong wheat flour as observed by Pyler (1988). The energy (W) required for deformation is an indication of the baking strength of the dough. It ranged from 175.8 (J x 10^{-4}) in CF to 160.3 (J x 10^{-4}). The farinographic characterization of all flour samples are shown in Table 2 (B) and Fig.1. Gluten Friendly Flour absorbed 10% more water than do CF when forming a dough.

A)

Alveograph				
Samples	**P (mm)**	**L (mm)**	**W (J x 10^4)**	**P/L**
CF	$52.0^{a} \pm 1.2$	$81.5^{a} \pm 0.3$	$175.8^{a} \pm 0.7$	$0.63^{a} \pm 1.5$
GFF	$164.6^{b} \pm 1.7$	$25.4^{b} \pm 0.4$	$160.3^{b} \pm 1.3$	$6.31^{b} \pm 1.7$

a, b= $p<0.05$

B)

Farinograph			
Absorption (%)	**Dough development time (min)**	**Dough stability (min)**	**Mixing tolerance index (BU)**
$57.2^{a} \pm 1.3$	$5.2^{a} \pm 0.2$	$8.6^{a} \pm 0.4$	$35.2^{a} \pm 0.5$
$63.1^{b} \pm 1.5$	$1.6^{b} \pm 0.6$	$2.1^{b} \pm 0.5$	$97.4^{b} \pm 0.6$

a, b= $p<0.05$

Table 2. Alveographic (A) and farinographic (B) characterization of Control Flours (CF) and Gluten Friendly Flours (GFF). Mean values ± standard deviation in the same column followed by a different letter are significantly different ($p\leq0.05$).

Water is responsible in hydrating the protein fibrils and facilitating the interaction between the protein crosslinks with with the disulfide bonds during dough mixing. An optimum amount of water is needed to develop cohesive, viscoelastic dough with optimum gluten strength. Rosell et al., (2001) reported that the differences in water absorption are caused predominantly by the greater number of hydroxyl groups that exist, which allows for more water interaction through hydrogen bonding. Indeed, breaking the hydrogen bonds between protein glutamines in wheat kernels induced by microwave treatment may be consistent with the increase in the percentage of water adsorbed by the flour during

mixing. In fact, if more glutamines are available to form hydrogen bonds with water for the plasticization of dough, a higher percentage of water must be added to the flour (Lamacchia et al., 2016). The dough development time (DDT) in GFF was significantly lower ($p<0.05$) than the CF.

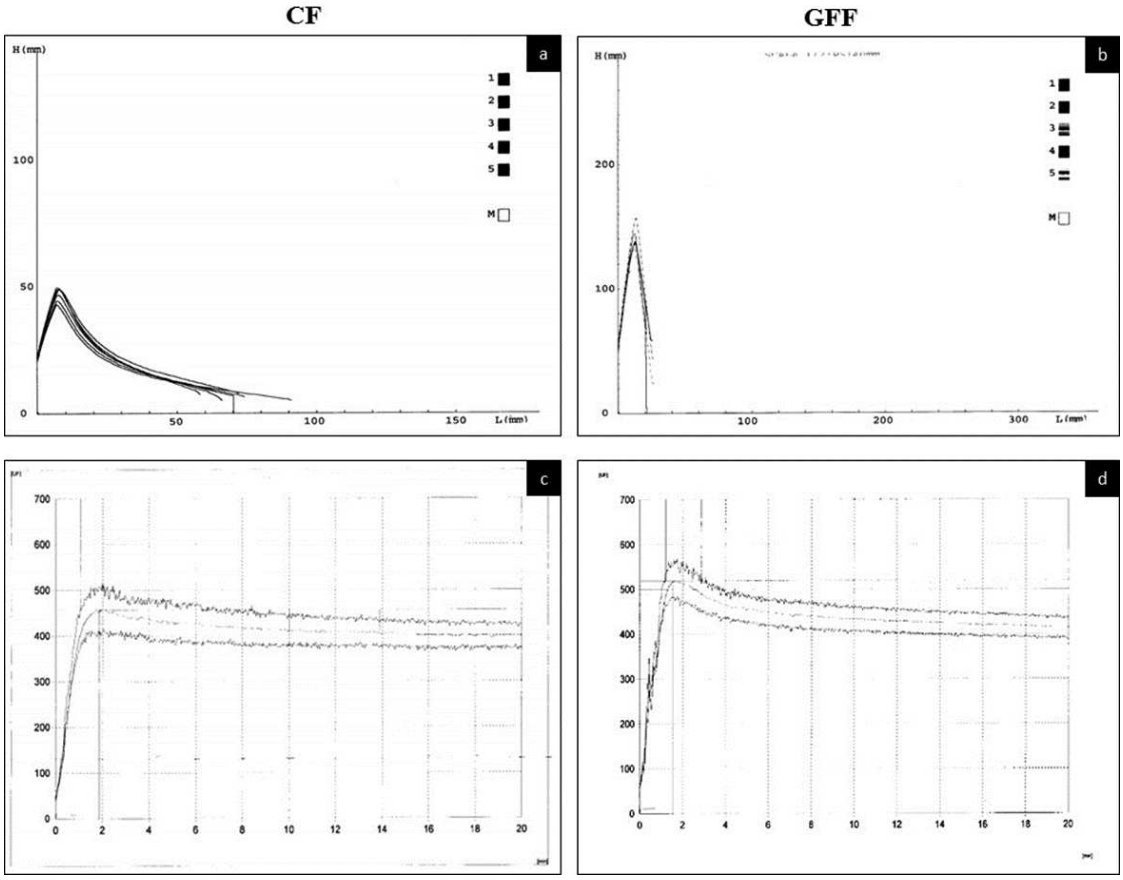

Figure 1. Alveogram and Farinogram of Control Flours (CF) (a,c) and Gluten Friendly Flours (GFF) (b,d).

The dough development time is an indication of potein quality; stronger flours normally require a longer development time than do weaker flours. Similarly the dough stability (DST) values in GFF were lower when compared to CF. Dough Stability Time (DST), indicated how much tolerance the flour has to over or under mixing (Schiller 1984). Mixing tolerance index (MTI) for Gluten Friendly Flour was significantly higher when compared to control flour. Generally, flours with good tolerance to mixing have low MTI; the higher the MTI value, the weaker the flour (Shuey 1990). These results indicate higher strength for CF which could be due to the presence of higher gluten content of CF compared to GFF. The extensiograph characteristics of the doughs prepared from wheat flour (CF) and wheat flour after treatment (GFF) is shown in Table 3 and Figure 2. Extensiograph gives information about the viscoelastic behavior of dough (Rosell et al., 2001). The extensograph measures the extensibility (E), the energy (cm^2), the resistance (BU) and the resistance to extension (R/E) (BU) at 45, 90 and 135 min resting time. There are remarkable differences in dough characteristics between

CF and GFF samples. From the results obtained, the energy of the dough (dough strength) and the dough extensibility were decreased in GFF samples when compared to CF.

		Samples	
		CF	**GFF**
Energy (cm^2)	45 min	$45.3^a \pm 1.2$	$45.1^a \pm 1.5$
	90 min	$45.2^a \pm 1.4$	$51.4^b \pm 1.2$
	135 min	$46.4^a \pm 1.1$	$62.5^b \pm 0.8$
Resistance to extension (BU)	45 min	$120.1^a \pm 0.5$	$420.3^b \pm 0.9$
	90 min	$126.3^a \pm 0.7$	$520.2^b \pm 0.3$
	135 min	$135.1^a \pm 0.9$	$615.5^b \pm 1.2$
Extensibility (mm)	45 min	$196.1^a \pm 0.4$	$81.2^b \pm 1.3$
	90 min	$191.4^a \pm 2.1$	$79.1^b \pm 1.6$
	135 min	$189.3^a \pm 1.4$	$77.2^b \pm 1.5$
Maximum Resistance (BU)	45 min	$148.1^a \pm 0.9$	$434.6^b \pm 0.5$
	90 min	$153.4^a \pm 1.2$	$547.3^b \pm 1.5$
	135 min	$161.2^a \pm 0.7$	$648.5^b \pm 0.3$

Table 3. Extensiograph evaluation of Control Flours (CF) and Gluten Friendly Flours (GFF). Mean values ± standard deviation in the same column followed by a different letter are significantly different ($p \leq 0.05$).

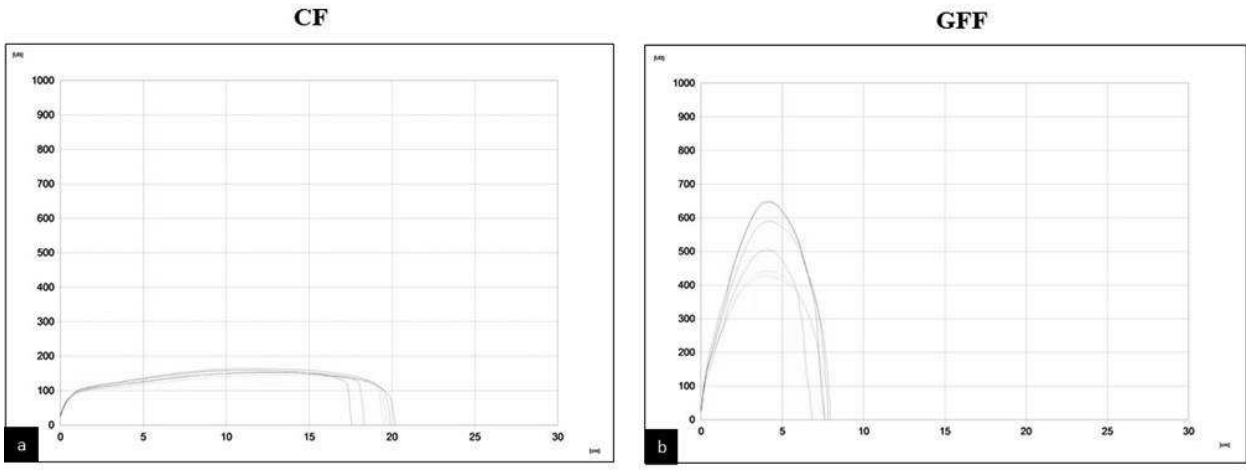

Figure 2. Extensiogram of Control Flours (CF) and Gluten Friendly Flours (GFF).

Although the precise nubers are somewhat arbitrary, flours that give extensigrams with areas less than 80 cm^2 can be classified as weak; those with areas of 80-120 cm^2 can be classified as medium; those with areas of 120-200 cm^2 can be classified as strong; and those with areas above 200 cm^2 can be classified as very strong (Preston & Hoseney, 1991). According to these classifications, Gluten Friendly Flour can be classified as medium and the Control wheat flour samples as strong. The significantly decrease ($p < 0.05$) in the elasticity of the treated flour samples is consistent with the observation that breaking the hydrogen bonds between gluten proteins and conformational changes

induced by microwave treatment in the kernel allow polymers in the flour to be hydrated but not to form a high amount of loop to train structures that are important for determining dough elasticity (Lamacchia et al., 2016).

3.3 Pasting properties of the dough

Amylograph results of wheat flour samples is shown in Table 4. The present work was aimed at studying the dynamics of gelatinization of Gluten Friendly Flour (GFF). Amylograph test measures the effect of alpha-amylase on the peak viscosity of a cooked flour slurry. The baking properties of flour mainly depend on the gelatinization of the starch and on the enzyme activity (α-amylase) in the flour. There were no significant differences in the values of maximum viscosity and gelatinization temperatures of tested wheat flours. Gelatinisation temperature is an important factor determining the technological quality of starch (Shuey and Tipples, 1994). Pasting temperature gives an indication of temperature required to cook flour beyond its gelatinization point (BeMiller 2011). Pasting temperature of wheat flour (beginning of gelatinization) ranged from 61.6 to 60.2°C, and gelatinization maximum (maximum viscosity in Amylograph unit) ranged from 995.4 to 1010.5 AU, for CF and GFF samples, respectively.

Generally it can be concluded that pasting temperature of Gluten Friendly Flours was not affected by the microwave treatment, and starch properties and functionality were also preserved.

These results are in accordance with previous research, indicating that starch granules did not differ in number and shape in Gluten Friendly Grain and Control Grain samples, moreover they were dispersed in the continuous protein matrix (Landriscina et al., 2017).

Samples	Initial temperature of gelatinization (°C)	Final temperature of gelatinization (°C)	Maximum Viscosity (AU)
CF	$61.6^{a} \pm 1.3$	$91.3^{a} \pm 0.7$	$995.4^{a} \pm 1.3$
GFF	$60.2^{a} \pm 0.9$	$89.5^{a} \pm 1.1$	$1010.5^{a} \pm 0.8$

Table 4. Pasting properties of Control Flours (CF) and Gluten Friendly Flours (GFF). Mean values ± standard deviation in the same column followed by a different letter are significantly different ($p \leq 0.05$).

3.4 Bread crumb image analysis

The structural elements of food products such as bread can be quantified by image analysis, contributing to the understanding of quality-related porperties such as texture. The cellular crumb structure of cereal-based products, such as bread is an important contributing factor to their textural properties (Zghal et al., 1999) and to the determination and quantification of sensory quality (Mondal & Data, 2008). It is a common agreement that good quality bread should have a high porosity and

fine, regular gas cell structure in the crumb (Liu and Scanlon, 2003; Lassoued et al., 2008). There were no significant differences ($p< 0.05$) between the image analysis parameters (numbers of cells, mean cell area and circularity) for the Gluten Friendly Bread (GFB) and Control Bread (CB) loaves, as shown in Table 5. The bread made with Gluten Friendly flour had significantly lower cell density than the control bread ($p<0.05$). Despite a difference in cell density, both samples exhibited a very compact structure (Fig. 3).

According to Polaki et al., (2010), the pore distribution reveals information about the effect on bread structure. The pores of the GFB and CB were characterized as small. These results suggests similar breadcumb texture properties as shown in Figure 3, for Gluten Friendly Bread when compared to Control Bread.

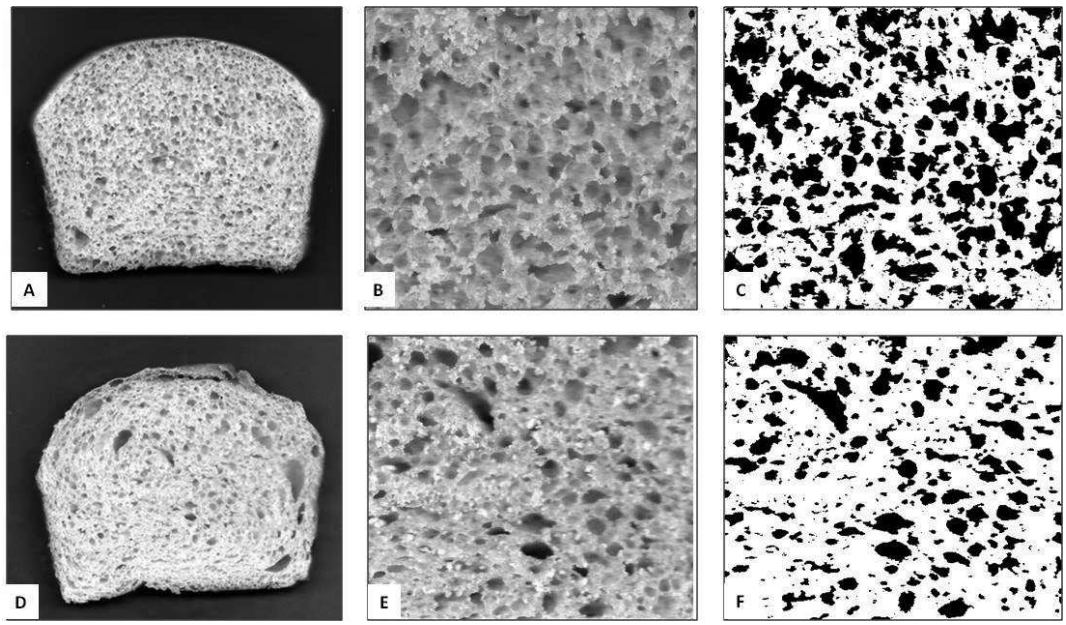

Figure 3. Breadcrumb digital and binary images (40mm x 40 mm crumb area) of Control bread (CB) and Gluten Friendly Bread (GFCB).

Bread samples	Number of cells	Mean cell area (mm^2)	Cell density ($cells/mm^2$)	Circularity
CB	$291.5^a \pm 0.9$	$0.129^a \pm 0.5$	$2259.6^a \pm 1.8$	$0.736^a \pm 0.8$
GFB	$254.4^a \pm 0.7$	$0.137^a \pm 0.6$	$1856.6^b \pm 1.1$	$0.697^a \pm 0.2$

Table 5. Image analysis parameters of bread loaves. Mean values ± standard deviation in the same column followed by a different letter are significantly different ($p\leq0.05$).

3.5 Sensory evaluation

Sensory evaluation of bread samples was undertaken and summarized in Table 6 and Fig.4. The appearance and tactile perception of bread texture seems to be a decisive criterion for consumers'

acceptability. How the crumb feels by touch or in the mouth is greatly influenced by the size or the structure of the crumb cells: finer, thin-walled, uniformly sized cells yield a softer and more elastic texture than coarse and thick-walled cell structures (Lassoued et al., 2008). *Gluten Friendly Bread* (GFB) was compared to *Control Bread* (CB) and *Gluten Free Commercial Bread* (GFCB). Results from sensory evalutation indicated that mean scores for all bread sample sensory attributes for *Gluten Friendly Bread* (GFB) and *Control Bread* (CB) were generally high and well overall rated by judges. Table 6 shows that GFB and CB were not significantly different from each other and have comparable ratings in terms of appearance, taste, aroma, color and bread texture. This suggests that bread preparad with Gluten friendly flour does not affect desirable sensory attributes of the bread, without changing its organoleptic values. Moreover, GFCB showed a significant decrease in the sensory ratings of bread when compared to GFB and CB.

Attributes	Control Bread	Gluten Friendly Bread	Gluten Free Commercial Bread
Appearance	7,51a	7,14a	5,98b
Crust colour	7,38a	7,27a	4,73b
Crumb colour	7,82a	6,89b	5,42c
Mouth feel	8,14a	7,78a	5,84b
Crumb stability	8,31a	7,14a	6,13b
Texture	7,92a	7,52a	2,78b
Taste	7,89a	7,95a	4,65b
Aroma	8,15a	8,36a	3,78b
Overall acceptability	7,45a	7,16a	5,39b

Table 6. Sensory evaluation of bread samples. Mean values ± standard deviation in the same row followed by a different letter are significantly different ($p \leq 0.05$).

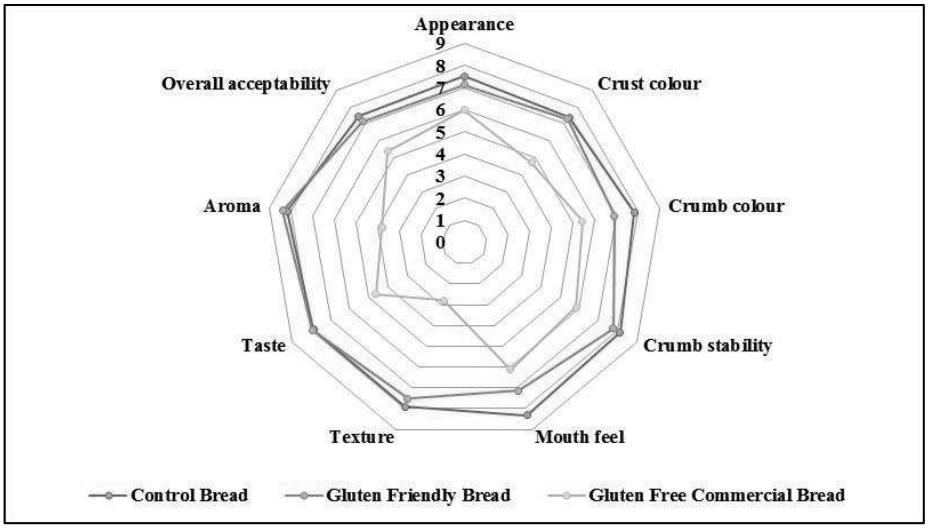

Figure 4. Radar plot of hedonic sensory evaluation of Control bread (CB), Gluten Friendly Bread (GFB) and Gluten Free commercial bread (GFCB).

However, the high technological value renders gluten almost indispensable in baked products, and its replacement, as structure-building protein, presents a major technological challenge for the food industry. Although many advances have been made in the preparation processes of gluten-free products, using starches, hydrocolloids, gums and novel ingredients (Zannini et al., 2012), many gluten-free industrial products available on the market exhibit a low nutritional quality, poor mouth feel or flavor (Arendt et al., 2002).

4. Conclusion

In this study, attempt was made to evaluate technological proterties of Gluten Friendly Flours in the production of bread. This was done by analyzing the rheological properties of the flour and dough, as well as baking qualities of the resulting bread. Gluten friendly Flour manifested significantly different rheological behaviour, such as farinograph water absorption, alveograph P/L ratio and also extensograph energy and resistance to extension. Despite significant changes in gluten properties, baking properties and sensory evaluation results obtained from this study has demonstrated the potential for the bread production from Gluten Friendly Flours.

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Chapter 5

Impact of Gluten-Friendly Bread on the Metabolism and Function of In Vitro Gut Microbiota in Healthy Human and Coeliac Subjects

Impact of Gluten-Friendly Bread on the Metabolism and Function of In Vitro Gut Microbiota in Healthy Human and Coeliac Subjects

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Abstract

The main aim of this paper was to assess the *in vitro* response of healthy and coeliac human faecal microbiota to gluten-friendly bread (GFB). Thus, GFB and control bread (CB) were fermented with faecal microbiota in pH-controlled batch cultures. The effects on the major groups of microbiota were monitored over 48 h incubations by fluorescence *in situ* hybridisation. Short-chain fatty acids (SCFAs) were measured by high-performance liquid chromatography (HPLC). Furthermore, the death kinetics of *Lactobacillus acidophilus*, *Bifidobacterium animalis* subsp. *lactis*, *Staphylococcus aureus*, and *Salmonella* Typhimurium in a saline solution supplemented with GFB or CB were also assessed. The experiments in saline solution pinpointed that GFB prolonged the survival of *L. acidophilus* and exerted an antibacterial effect towards *S. aureus* and *S.* Typhimurium. Moreover, GFB modulated the intestinal microbiota *in vitro*, promoting changes in lactobacilli and bifidobacteria members in coeliac subjects. A final multivariate approach combining both viable counts and metabolites suggested that GFB could beneficially modulate the coeliac gut microbiome; however, human studies are needed to prove its efficacy.

Introduction

Coeliac disease is a chronic immune-mediated enteropathy triggered by the ingestion of gluten in HLA-DQ2- or HLA-DQ8-positive people. Approximately 30% of the general population carries the HLA-DQ2/8 coeliac disease susceptibility genes; however, only 2–5% of these individuals will go on to develop coeliac disease, suggesting that additional environmental factors contribute to the disease development [1]. Many authors have reported that coeliac people suffer from an altered composition of gut microbiota [2,3], such as lower levels of Bifidobacterium spp. and Lactobacillus spp. [4,5]; higher levels of Bacteroides, Escherichia coli, Staphylococcus and Clostridium [4,6–8]; and an altered profile of short-chain fatty acids (SCFAs) [4,9]. The link between an altered gut microbiota composition and disease is a matter of debate, as some authors have suggested that this change could be both an effect of disease and the cause of some symptoms [10]. To date, the only treatment for coeliac patients is the complete lifelong exclusion of gluten from the diet, but a gluten-free diet does not completely restore healthy microbiota profiles [11]. An alternative to the exclusion of gluten is its detoxification without affecting the technological performance of the flour and dough. Some approaches for gluten detoxification have been proposed and studied in the past, e.g. the use of protease produced by lactic acid bacteria [12] or the transdamidation [13]. Recently, we developed a new and innovative method to detoxify gluten proteins from cereal grains with the purpose of combining the nutritional and technological properties of wheat proteins with safety for coeliac and gluten-sensitive patients [14,15]. This innovation is usually referred to as “gluten friendly” and relies on 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 and induce a structural change in gluten proteins [16]. This modification abolishes the antigenic capacity of gluten [16] and reduces in vitro the immunogenicity of the most common epitopes involved in coeliac disease [17], without compromising the nutritional and technological properties necessary to process semolina in pasta and flours in bread and other baked goods [16]. Additionally, the technology has been further improved [18]. Scaling up a new technology is a complex process because many issues must be addressed. For a gluten-friendly approach, we have only assessed the effect of microwave on gluten proteins [16], and the industrial scale-up is in progress. This paper addresses the effect of gluten-friendly bread (GFB) on the gut microbiota composition through two intermediate steps:

Assessing the effect on certain foodborne strains (both probiotic and pathogenic) to pinpoint whether the addition of GFB modifies the survival of these selected targets under strict controlled conditions. This step was necessary to select and/or design an experiment evaluating the effects of GFB in a complex system;

Investigating the effect of GFB on faecal human microbiota in healthy and coeliac subjects in a pH-controlled, stirred, batch-culture fermentation system that is reflective of the environmental conditions of the distal region of the human large intestine, with a focus on the viable count of certain microbial groups and the production of SCFAs.

Materials and Methods

Raw materials and microwave treatment

The Casillo group S.p.a (Corato, Italy) supplied the wheat kernels (mixtures of soft wheat Canadian grains; the exact composition of the mixtures was not specified). Flour treated with microwaves was called gluten-friendly flour (GFF) and was obtained by milling the microwave-treated caryopses [18]. One hundred grams of cleaned wheat grains were 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 (Delonghi, Italy, approximately 1 min. 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, i 20 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, Azwil, Switzerland, diameter of grid 118–180 μm). 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.

Bread production and digestion

Bread was produced by using either CF (control bread, CB) or GFF (GFB) according to the Chorleywood Bread Process in the Food Processing Centre of the Department of Food and Nutritional Sciences at the University of Reading (UK). Bread was prepared as follows: flour, 100 g; water, 66 mL; baker's yeast, 1.33 g; and salt, 1 g. Allinson Easy Bake Yeast (UK) has been used as a dried yeast with the bread improver Ascorbic Acid (Vitamin C).

Simulated in vitro human digestion

Bread was digested in vitro under appropriate conditions according to the procedures described by Maccaferri et al. [19] in order to mimic mouth, stomach and intestine's condition. The only

modification to the method described by Macaferri et al. [19] was to not apply any form of dialysis to the samples.

In vitro experiments

Microorganisms

Lactobacillus acidophilus La-5, Bifidobacterium animalis subsp. lactis Bb-02, Salmonella Typhimurium and Staphylococcus. aureus were used throughout this research. L. acidophilus and B. animalis were purchased from Chr. Hansen and stored at -20°C in MRS broth (Oxoid, Milan, Italy) supplemented with 33% sterile glycerol (J.T. Baker, Milan). The pathogens were food-borne isolates belonging to the culture collection of the Laboratory of Predictive Microbiology, University of Foggia; the strains were stored at 4°C on tryptone soya agar slants (Oxoid).

Before each assay, the microorganisms were grown at 37 °C for 24 h in the optimal media (MRS broth or TSB broth); the cultures were centrifuged two times at 1000 g for 10 min, and the cells were suspended in sterile distilled water.

Samples and microbiological analyses

Two different sets of experiments were performed, as reported in Table 1. Aliquots of saline solution (0.9% NaCl) (50 mL) were supplemented with different amounts of dried CB or dried GFB and inoculated to approximately 8 log CFU mL-1; the samples were periodically analysed to assess the viable count by plating on MRS agar (L. acidophilus and B. animalis) or TSA (Salmonella sp., S. aureus) and incubating at 37 °C for 2–4 days. L. acidophilus and B. animalis were assessed under anaerobic conditions

Modelling and statistics

The experiments were performed on two different batches in duplicate (n = 2). The results of the viable count were fitted using the Weibull equation in the log-linear form [20]:

$$\log N = \log N0 - (t/\delta)p$$

where log N is the cell count over time (log CFU mL-1); log N0 is the initial cell count; δ is the first reduction time (day), i.e., the time to attain a decrease in cell count of 1 log CFU mL-1; and p is the shape parameter ($p>1$, downward curve; $p<1$, upward curve).

The data were also modelled through Weibull equation, as modified by Bevilacqua et al. [21], to evaluate the death time of the population.

The fitting parameters of the Weibull equation, as well as the viable counts, were analysed by one-way analysis of variance (ANOVA) and Tukey's test as the post-hoc comparison test ($P<0.05$). Statistics were performed in Statistica software for Windows (StatSoft, Tulsa, OK).

Experiments	Targets	Samples	Duration
Death kinetics	*L. acidophilus B. animalis*	Saline solution and samples supplemented with 0.4 or 0.8 g L^{-1} of either CB or GFB*	7 days (viable count every 6–10 h)
Effect of concentration	*L. acidophilus B. animalis*	Saline solution and samples supplemented with 0.8 or 5.0 g L^{-1} of either CB or GFB	24 h
Pathogens	*Salmonella* Typhimurium *S. aureus*	Saline solution and samples supplemented with 0.2, 0.4, or 0.8 g L^{-1} of either CB or GFB	7 days (viable count after 1 and 7 days)

*CB, control bread; GFB, gluten friendly bread.

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Table 1. Conditions used to assess the effect of control bread and gluten friendly bread towards L. acidophilus, B. animalis, Salmonella sp. and S. aureus in saline solution.

Batch culture fermentations

Previously sterilized batch culture fermentation vessels (280 mL working volume) were filled with 45 mL of sterile complex colonic model growth medium. The composition of this medium included peptone water (5 g L-1), yeast extract (4.5 g L-1), starch (5 g L-1), tryptone (5 g L-1), NaCl (4.5 g L-1), KCl (4.5 g L-1), mucin (4 g L-1), casein (3 g L-1), pectin (2 g L-1), xylan (2 g L-1), arabinogalactan (2 g L-1) and inulin (1 g L-1) [22]. All media and chemicals were purchased from Oxoid and Sigma. Then, the vessels were connected to a circulating water bath at 37°C and sparged with O2-free N2 gas overnight to attain anaerobic conditions. The pH was adjusted to between 6.7 and 6.9 using pH meter controllers with NaOH or HCl (Electrolab260; Electrolab Ltd., Tewkesbury, UK), and 5 mL of faecal slurry was then inoculated in each vessel. In total, eighteen vessels were prepared and supplemented with 1 mL of CB or GFB digesta (3 vessels per type of donor, including a negative control, i.e., a sample containing faecal slurry but without bread, and samples with CB and GFB). The batch cultures were run for 48 h, and 5 mL of the samples were removed at 0, 6, 24 and 48 h for analysis of bacterial populations by fluorescence in situ hybridisation (FISH) and SCFA analysis by high-performance liquid chromatography (HPLC).

Enumeration of bacterial populations by fluorescence in situ hybridisation (FISH)

FISH was performed as described by Costabile et al. [23]. A 375 µL aliquot of the batch culture samples was fixed in three volumes of ice-cold 4% (w/v) paraformaldehyde for 4 h at 4°C, centrifuged at 13,000 g for 5 min and washed twice in 1 mL of sterile PBS. The cells were again pelleted by centrifugation and re-suspended in 150 µL of sterile PBS, to which 150 µL of ethanol was added. The samples were then mixed and stored at -20°C until used.

All probes were synthesised by Sigma-Aldrich. The following bacterial groups were identified using synthetic oligonucleotide probes that target specific regions of the 16S ribosomal RNA molecule, labelled with the fluorescent dye Cy3: Clostridium hystolyticum clusters I/II (Chis150, TTATGCGGTATTAATCTYCCTTT) [24], Lactobacillus/Enterococcus spp. (Lab158, GGTATTAGCAYCTGTTTCCA) [25], Clostridium clusters XIVa+b (Erec482, GCTTCTTAGTCARGTACCG) [24], Bacteroides/Prevotella group (Bac303, CCAATGTGGGGGACCTT) [26], Bifidobacterium spp. (Bif164, CATCCGGCATTACCACCC) [27] and Eub338 I-II-III (GCTGCCTCCCGTAGGAGT, GCAGCCACCCGTAGGTGT, GCTGCCACCCGTAGGTGT) [28].

Short-chain fatty acid (SCFA) analysis

Samples were taken from the batch culture vessels at each time point, and cell-free culture supernatants were obtained by centrifuging 1 mL at 13000 x g for 10 min, followed by filter sterilisation (0.2 μm Acrodisc® syringe filters with a hydrophilic polyvinylidene fluoride (PVDF) membrane, 13 mm; Pall Corporation) to remove particulate matter. The SCFA content was quantified on an ion exclusion HPLC (LaChrom Merck Hitachi, Poole, Dorset, UK) instrument equipped with a pump (L-7100), RI detector (L-7490) and autosampler (L-7200). Samples (20 μL) were injected into the HPLC at a flow rate of 0.5 mL min-1 with a prepacked Rezex ROA–Organic Acid H+ 80% (300 x 7.8 mm) column at 84°C and a detector wavelength of 210 nm. H2SO4 (2.5 mM) was used as the eluent, and the organic acids (lactic, acetic, propionic and butyric) were calibrated against standards at concentrations of 12.5, 25, 50, 75 and 100 mM. An internal standard of 2-ethylbutyric acid (20 mM) was included in the samples and external standards. All chemicals were provided from Sigma-Aldrich.

Statistical analysis

The results from the FISH and SCFA analyses were standardized and reported as increases/decreases relative to t0 of the negative control (beginning of the experiment). The results were first analysed through one-way ANOVA and Tukey's test using the homogeneous group approach [29]. The classical approach of one-way ANOVA and post-hoc offers an overview of the significant differences amongst different samples. However, it does not work well if the studied parameter does not show or possess well-defined statistical groups and is distributed in a continuous way and if each sample can be attributed to different statistical groups. When this type of distribution is observed in the results, the use of ANOVA by homogeneous groups is advisable: the parameters are organized in a table with different columns (homogeneous groups), and each column contains the samples belonging to the

same homogeneous group. The novelty relies upon the fact that the same sample can be attributed to many groups. Thereafter, the FISH and SCFA values were used as input data to run 3 different principal component analysis (PCA; for the results after 6, 24, and 48 h) experiments.

As a confirmatory experiment, a second standardization of the data was performed, i.e., for each sampling time, the results from healthy and coeliac donors supplemented with CB or GFB were reported as increases/decreases relative to the negative control at the same time, and the results were then analysed through one-way ANOVA. This type of modelling was used to exclude possible prebiotic activities of CB or GFB on both healthy and coeliac people.

Sample	log N_0*	δ	p	d.t.	R
CB 0.4 g L^{-1}	8.43±0.14A	17.99±0.90A	1.62±0.15A	67.46±2.06A	0.995
GFB 0.4 g L^{-1}	8.38±0.13A	17.43±2.06A	1.40±0.14A	80.53±2.03B	0.994
CB 0.8 g L^{-1}	8.19±0.12A	23.40±2.00B	1.94±0.20A	70.28±2.63A	0.993
GFB 0.8 g L^{-1}	8.56±0.14A	17.57±2.70A	1.27±0.17A	93.96±4.00C	0.990

*log N_0, initial cell count (log CFU mL^{-1}); δ, first reduction time (h); p, shape parameter; d.t., death time (h).

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Table 2. Fitting parameters of the Weibull equation for the death kinetics of L. acidophilus (mean values ± SE). For each parameter, the letters indicate significant differences (one-way ANOVA and Tukey's test, P<0.05. CB, control bread; GFB, gluten friendly bread.

Results

In vitro experiments

Table 2 reports the fitting parameters for L. acidophilus; the death kinetics generally showed a downward curve with a shape parameter of the Weibull equation >1. The supplementation of saline solution with either CB or GFB did not affect the shape of the curve. On the other hand, the type of bread exerted a significant effect on the death time of the bacterial population; in fact, the death time was prolonged from 67.46 (CB) to 80.53 (GFB) at 0.4 g L-1 and from 70.28 (CB) to 93.96 (GFB) at 0.8 g L-1. The effect of GFB on the death time, but not on the shape parameter, was a consequence of a probable reduction of the death rate in the last part of the death curve, as suggested by the kinetics reported in Fig 1. The death kinetic of B. animalis subsp. lactis was not affected by the type of supplementation (S1 Table).

A second assay was run to determine whether the concentration of GFB could cause or exert a detrimental effect on both L. acidophilus and B. animalis; saline solution was supplemented with same the amount used in the first experiment (0.8 g L-1) and with a higher concentration (5.0 g L-1). The viable count was not affected by the concentration of bread digesta (S2 Table).

Finally, saline solution was inoculated with a Gram-positive or a Gram-negative pathogen (S. aureus and Salmonella Typhimurium); the results for S. aureus are shown in Fig 2. A significant difference was found for the sample supplemented with 0.8 g L-1 GFB, which showed a 1-log lower viable

count than the sample supplemented with CB. Salmonella sp. experienced a 1.8- and 2.84-log reduction after 6 and 8 days, respectively (Fig 3).

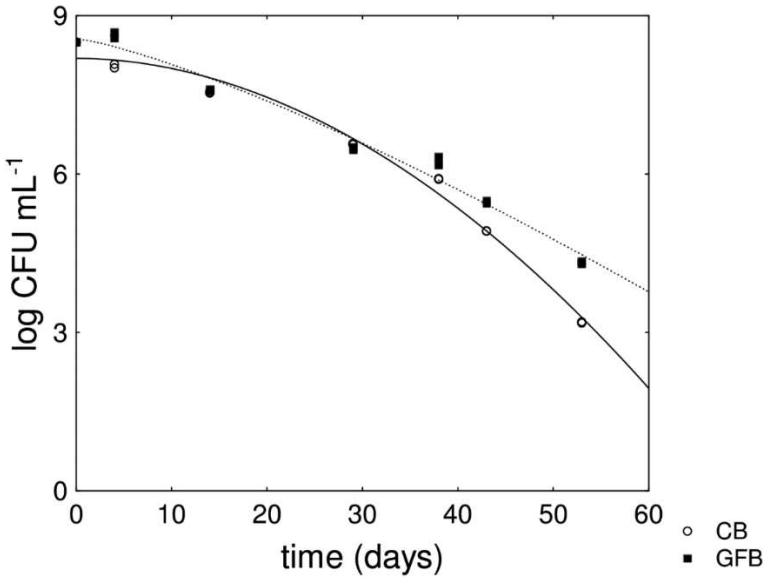


Fig 1. Death kinetics of L. acidophilus in a saline solution supplemented with either control (CB) or gluten-friendly bread (GFB) (0.8 g L-1). The lines represent the best fit through the Weibull equation. doi:10.1371/journal.pone.0162770.g00

Changes in bacterial populations and the metabolic profile with in vitro batch culture fermentation: a one-way ANOVA approach.

A second step was aimed at assessing the effect of both CB and GFB in batch culture fermentations. See S1 and S2 Figs for the raw data.

The results of the FISH and SCFA analyses were standardized to the negative control at time 0 (inoculation time) to exclude the variability due to the type of donor; thus, the results show the modification of the system compared to the beginning of the experiment and should be read as increases (positive values) or decreases (negative values). Moreover, each parameter was analysed through one-way ANOVA to pinpoint significant differences; the homogeneous group approach was used as an additional tool to uncover a possible trend through time.

Table 3 shows the results for bifidobacteria. The differences amongst the samples were not significant after 6 h or 24 h, but two statistical groups were recovered after 48 h, i.e., sample E (coeliac donor with CB) and the other samples. Sample E did not show a significant increase in the viable count of bifidobacteria (0.27-log increase) probably due to a detrimental effect exerted by bread on the microbiota. In the other samples, there was a 0.7–09 log increase, and sample F (coeliac donor with GFB) experienced a similar trend to that of the samples from healthy donors.

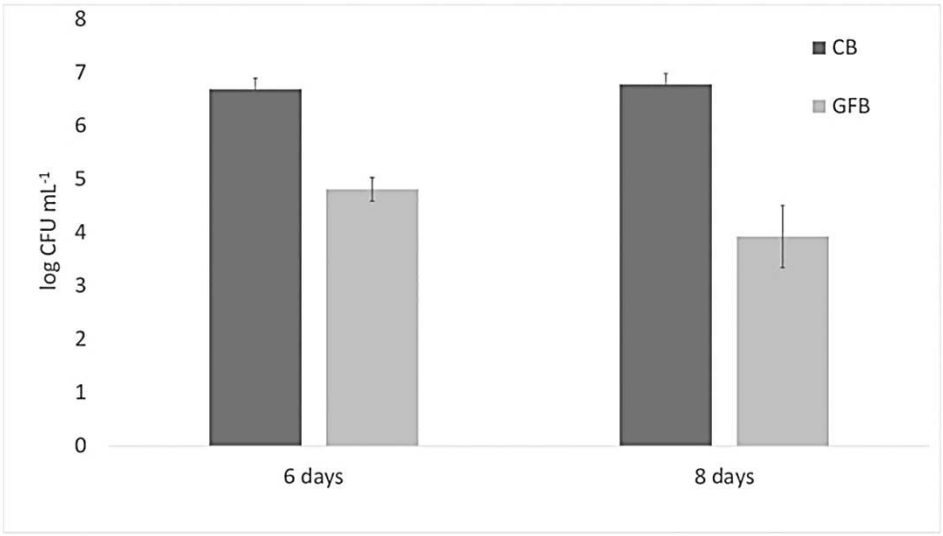


Fig 2. Viable count of S. aureus in a saline solution supplemented with either control (CB) or glutenfriendly bread (GFB) (0.2, 0.4 or 0.8 g L-1). Mean values ± standard deviation. The symbols "*" and "**" denote significant differences (one-way ANOVA and Tukey's test). doi:10.1371/journal.pone.0162770.g002

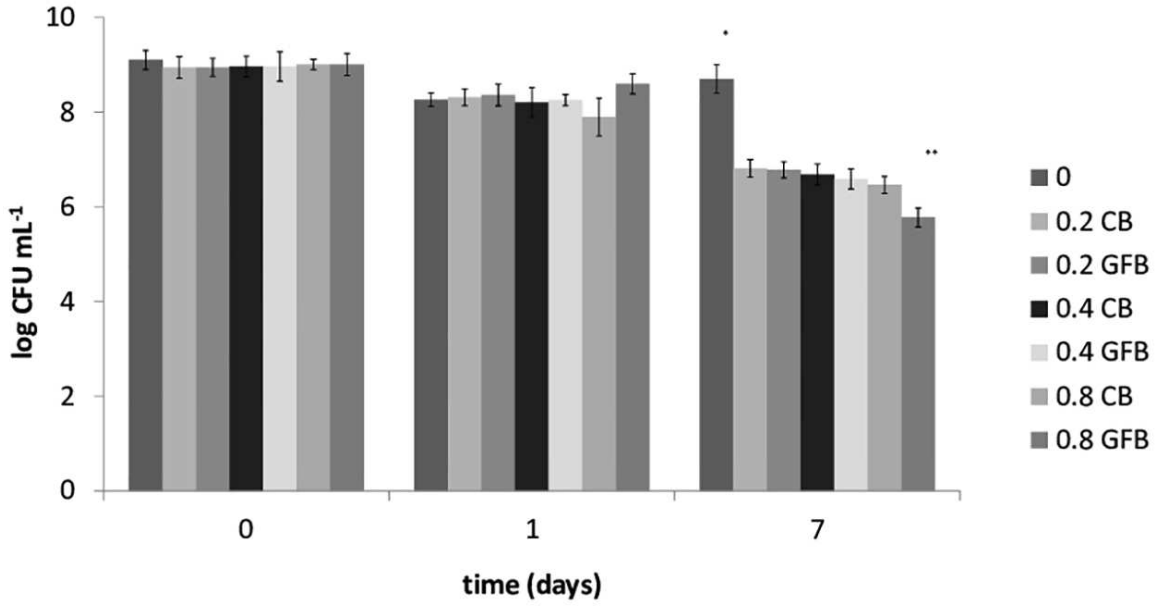


Fig 3. Viable count of Salmonella typhimurium in a saline solution supplemented with either control (CB) or gluten-friendly bread (GFB) (0.8 g L-1). Mean values ± standard deviation. doi:10.1371/journal.pone.0162770.g003

	FISH (log cells mL^{-1})	Homogeneous groups		
6 h		**I**	**II**	**III**
Sample				
E	0.15			
F	0.26			
D	0.49			
B	0.60			
A	0.63			
C	0.70			
24 h				
E	0.37			
F	0.49			
D	0.51			
A	0.72			
B	0.73			
C	0.91			
48 h				
E	0.27			
A	0.65			
F	0.68			
D	0.70			
B	0.72			
C	0.93			

doi:10.1371/journal.pone.0162770.t003

Table 3. One-way ANOVA (P<0.05) and homogeneous groups based on FISH data for bifidobacteria (enumerated as Bif164) after 6, 24 and 48 h of fermentation in pH-controlled batch culture systems. Increases/decreases refer to the inoculum of the negative control. Samples: A, negative control healthy donors; B, healthy donors + CB; C, healthy donors + GFB; D, negative control coeliac donors; E, coeliac donors + CB; F, coeliac donors + GFB. CB, control bread; GFB, gluten-friendly bread.

Concerning the outputs for the E. rectale–C. coccoides group (enumerated as Erec482) and the Bacteroides spp.–Prevotella group (enumerated as Bac303), the statistics highlighted a continuous rather than discrete distribution of the samples, with 2–4 overlapping homogeneous groups depending on the time and the type of microorganisms present. The sample distribution of Bacteroides/Prevotella changed over time; however, the increase/decrease in the absolute values of the viable count (-0-33-0.26 log CFU mL-1) was slight (S3 Table).

Table 4 shows the effect of bread supplementation on the numbers of the C. hystolyticum group (Chis150). After 6 h, there was a continuous distribution of samples with 2 well-defined groups (first group containing samples A and B; second group containing sample E) and an intermediate class (samples C, D, and F). Thus, sample E (coeliac donor with CB) was not different from samples D and F (negative control and coeliac donor with GFB), although it was significantly different from the healthy donor samples. Samples F and D experienced a slight statistical shift towards sample C. This shift was not observed after 24 and 48 h, suggesting that this result should be confirmed with prolonged experiments and repeated supplementation.

	FISH (log cells mL^{-1})	Homogeneous group		
6 h		**I**	**II**	**III**
Sample				
B	-0.27			
A	-0.18			
C	-0.10			
D	0.15			
F	0.17			
E	0.32			
24 h				
C	-0.16			
F	-0.12			
A	-0.08			
B	-0.03			
E	0.07			
D	0.10			
48 h				
A	-0.31			
C	-0.23			
B	0.06			
D	0.17			
F	0.19			
E	0.22			

doi:10.1371/journal.pone.0162770.t004

Table 4. One-way ANOVA (P<0.05) and homogeneous groups based on fluorescence in situ hybridization (FISH) data for the C. hystolyticum group (enumerated as Chis150) after 6, 24 and 48 h of fermentation in pH-controlled batch culture systems. Increases/decreases refer to the inoculum of the
negative control. Samples: A, negative control healthy donors; B, healthy donors + CB; C, healthy donors + GFB; D, negative control coeliac donors; E, coeliac donors + CB; F, coeliac donors + GFB. CB, control bread; GFB, gluten-friendly bread.

The lactic acid bacteria exhibited a characteristic trend over time, as reported in Table 5. The 6 h fermentation caused a decrease in the numbers of Lactobacillus/Enterococcus spp. in samples E and F (0.57–0.64 log CFU mL-1). After 24 h, the decrease was again observed in sample E but not in sample F, which exhibited a trend similar to the samples from healthy donors (no decrease in the cell count). After 48 h, the samples were distributed in a continuous way, and sample F was included in the group of healthy donors, as well as in the same group as sample E, suggesting that the shift observed after 24 h of fermentation was reversible. The statistical outputs for E. rectale pinpoint a constant distribution, without significant differences amongst the different samples (S4 Table).

The same approach was used to analyse the SCFA profiles (Tables (Tables6,6, ,77 and and8).8). The amount of SCFAs generally had a discrete distribution with well-defined statistical groups and significant differences. Acetate increased by 174 mM in sample B after 24 h, whereas the increase was 43–62 mM in the other samples. A similar trend was observed at the end of the assay, although a further increase was found (Table 6).

	FISH (log cells mL^{-1})	Homogeneous group		
6 h		**I**	**II**	**III**
Sample				
F	-0.64		■	
E	-0.57		■	
D	-0.33	■	■	
C	-0.12	■		
A	0.00	■		
B	0.04	■		
24 h				
E	-0.59		■	
F	0.01	■		
D	0.02	■		
A	0.17	■		
C	0.27	■		
B	0.29	■		
48 h				
E	-0.53			■
D	-0.29	■		■
F	-0.02	■	■	■
A	0.14	■	■	
B	0.19	■	■	
C	0.30		■	

doi:10.1371/journal.pone.0162770.t005

Table 5. One-way ANOVA (P<0.05) and homogeneous groups based on FISH data for Lactobacillus/ Enterococcus (enumerated with Lab158) after 6, 24 and 48 h of fermentation in pH-controlled batch culture systems. Increases/decreases refer to the inoculum of the negative control. Samples: A, negative control healthy donors; B, healthy donors + CB; C, healthy donors + GFB; D, negative control coeliac donors; E, coeliac donors + CB; F, coeliac donors + GFB. CB, control bread; GFB, gluten-friendly bread.

	Acetate (mM)	Homogeneous groups					
24 h		**I**	**II**	**III**	**IV**	**V**	**VI**
Sample							
C	43.37		■				
A	45.39			■			
D	52.36	■					
F	52.38	■					
E	62.30				■		
B	174.10					■	
48 h							
A	43.86	■					
F	52.96		■				
E	57.66			■			
D	61.84				■		
C	62.46					■	
B	258.47						■

doi:10.1371/journal.pone.0162770.t006

Table 6. One-way ANOVA (P<0.05) and homogeneous groups based on acetate after 24 and 48 h of fermentation in pH-controlled batch culture systems. Increases/decreases refer to the inoculum of the negative control. Samples: A, negative control healthy donors; B, healthy donors + CB; C, healthy donors + GFB; D, negative control coeliac donors; E, coeliac donors + CB; F, coeliac donors + GFB. CB, control bread; GFB, gluten-friendly bread.

	Propionate (mM)	Homogeneous groups					
24 h		I	II	III	IV	V	VI
Sample							
B	-4.31						
A	-0.76						
C	0.97						
D	23.16						
E	31.19						
F	32.71						
48 h							
A	0.39						
B	1.72						
C	4.69						
D	22.59						
F	37.14						
E	37.46						

doi:10.1371/journal.pone.0162770.t007

Table 7. One-way ANOVA (P<0.05) and homogeneous groups based on propionate after 24 and 48 h of fermentation in pH-controlled batch culture systems. Increases/decreases refer to the inoculum of the negative control. Samples: A, negative control healthy donors; B, healthy donors + CB; C, healthy donors + GFB; D, negative control coeliac donors; E, coeliac donors + CB; F, coeliac donors + GFB. CB, control bread; GFB, gluten-friendly bread.

	Propionate (mM)	Homogeneous groups					
24 h		I	II	III	IV	V	VI
Sample							
B	-4.31						
A	-0.76						
C	0.97						
D	23.16						
E	31.19						
F	32.71						
48 h							
A	0.39						
B	1.72						
C	4.69						
D	22.59						
F	37.14						
E	37.46						

doi:10.1371/journal.pone.0162770.t007

Table 8. One-way ANOVA (P<0.05) and homogeneous groups based on butyrate after 24 and 48 h of fermentation in pH-controlled batch culture systems. Increases/decreases refer to the inoculum of the negative control. Samples: A, negative control healthy donors; B, healthy donors + CB; C, healthy donors + GFB; D, negative control coeliac donors; E, coeliac donors + CB; F, coeliac donors + GFB. CB, control bread; GFB, gluten-friendly bread.

The increase in propionic acid was slight in the samples from healthy donors (after 24 and 48 h), whereas the content of this acid increased by 23–37 mM in the samples from coeliac donors (Table 7). After 24 h, butyric acid increased by 17 mM in the negative control D, which experienced the highest increase, followed by the other two samples from the coeliac donors (E, 7.6 mM; F, 4.1 mM). The results after 48 h revealed an interesting output; sample E experienced a further increase in butyric acid of 3 mM, and an increase was found in samples A (2 mM), B (4 mM) and C (6.58 mM); sample F showed a profile similar to the samples from healthy donors, with a net increase in butyric acid of 4.28 mM (Table 8).

Changes in the bacterial populations and metabolic profile with in vitro batch culture fermentation: a multivariate approach

The results from the batch cultures were analysed through PCA. SCFA and FISH data were all used as input, except for the data on the C. hystolyticum and E. rectale–C. coccoides groups due to the results of one-way ANOVA (no significant differences). Three analyses were run in order to assess the factorial distribution of the samples as a function of the origin (coeliac or healthy donors), supplementation (CB or GFB) and time (6, 24, and 48 h), as shown in Figs Figs44–6. For each analysis, we reported both the variable (Figs (Figs4A,4A, ,5A5A and and6A)6A) and case distribution (Figs (Figs4B,4B, ,5B, 5B and 6B); 6B); however, we did not use PCA to discuss why and for which type of variable the samples were different (this topic was addressed in the previous section). We used PCA to graphically estimate the similarity or dissimilarity amongst the samples. After 6 h, 2 statistical groups could be determined in the multi-factorial space, i.e., the first group including the samples from healthy donors (A, B, C) and the second group including the samples from coeliac donors supplemented with CB or GFB (E and F). The negative control of this latter group was an outsider and placed in a different region of the factorial space (Fig 4).

After 24 h, the space distribution drastically changed, and many samples experienced a shift in the plot (Fig 5); the group composed of healthy donors was divided in two sub-groups because sample B had moved from a different region of the space. A split was also found for the samples from coeliac donors: both E and F moved away from the negative control, but F moved in the upper region of the space towards samples A and C. A similar distribution was found after 48 h, except that samples B and E were in the positive quadrants of factor 2 (upper region) and the other samples were in the negative region (Fig 6).

The role of gut microbiota in health and well-being has been extensively reviewed [30]. Lifestyle, diet, life stage and some pathologies can strongly affect the qualitative and quantitative composition

of gut microbiota [31,32]. Coeliac people possess altered gut microbiota; in addition, many researchers pinpointed that dysbiosis dramatically impacts the host physiology [33].

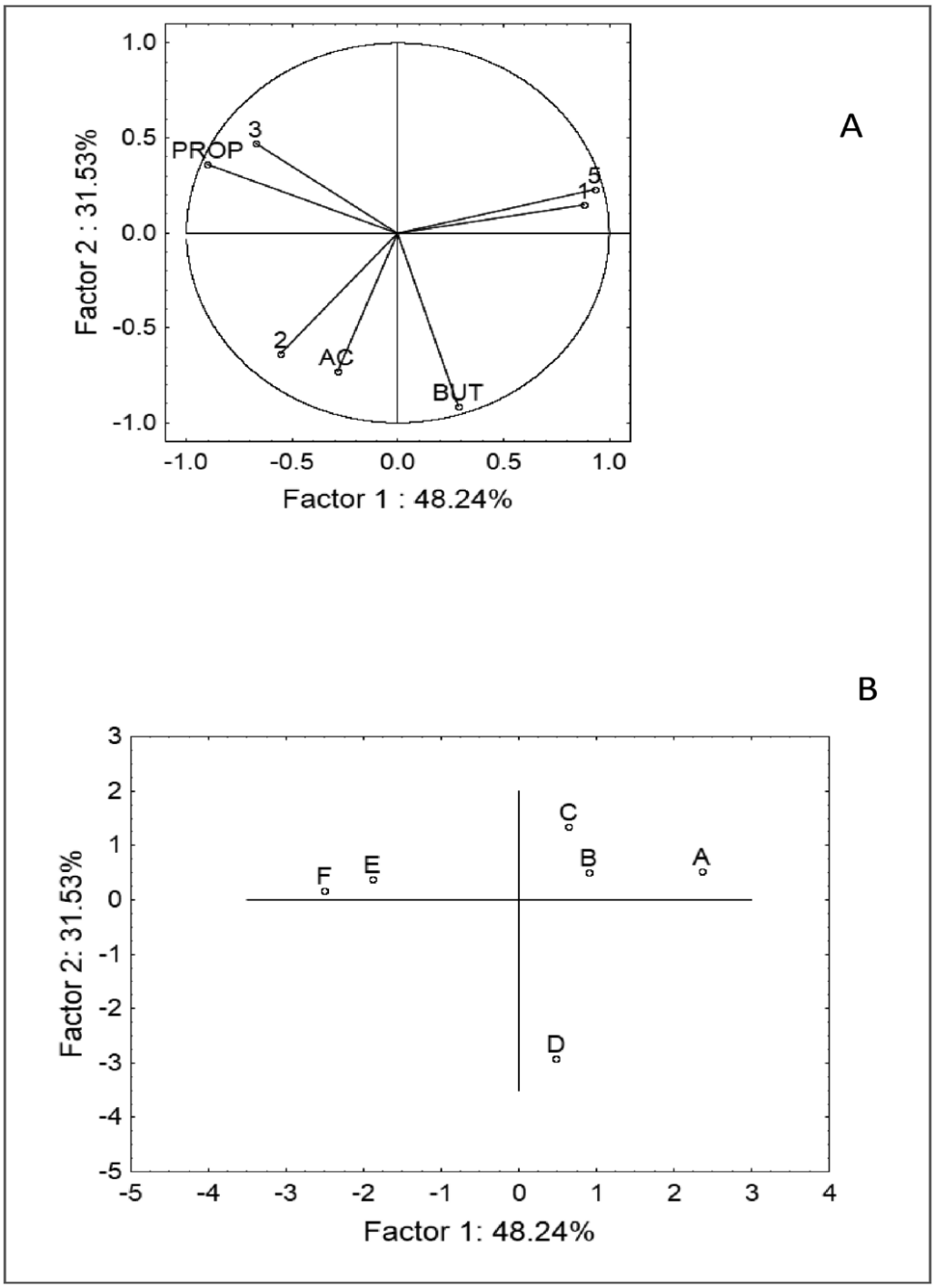


Fig 4. PCA based on the SCFA and FISH data after 6 h of fermentation. A) Variable projection; B) Case projection. Samples: A, negative control healthy donors; B, healthy donors + CB; C, healthy donors + GFB; D, negative control coeliac donors; E, coeliac donors + CB; F, coeliac donors + GFB. CB, control bread; GFB, gluten-friendly bread. Variables: 1, bifidobacteria; 2, E. rectale-C. coccoides; 3, Bacteroides/Prevotella; 5, Lactobacillus/Enterococcus; AC, acetate; BUT, butyrate; PROP, propionate.
doi:10.1371/journal.pone.0162770.g004

The background of this research was a patent [14,15] focused on the use of microwave for the detoxification of gluten. The safety of this technology was preliminary assessed on peripheral blood mononuclear cells (PBMC) stimulated with phytohaemagglutinin (PHA). The patent was further improved in terms of combination of microwave power, treatment time and rest time [18]; however, the effect of this approach on the microbiota of the gut has been never addressed and this topic has been investigated in the present paper.

Discussion

Firstly, we aimed to determine whether GFB modifies the qualitative-quantitative composition of gut microbiota via a two-step plan. The first phase relied on the evaluation of the death decay of selected strains and focused on the eventual change of shape or kinetics of the characteristic curve of each microorganism. For this step, the Weibull function pinpointed some interesting results. Namely, the Weibull equation possesses two fitting parameters (δ and p for the first reduction time and the shape parameter, respectively), which take into account two different phenomena of death kinetics, i.e., the prolongation of the shoulder phase (a benefit in the first step of the kinetics) and tailing (i.e., prolonged survival). As an additional tool, we used a slightly modified Weibull equation to evaluate death (or survival time). This last parameter is generally affected by the shoulder and tail, as well as the death rate. Supplementation with GFB affected neither the first reduction time nor the shape parameter of L. acidophilus but exerted a significant effect on the death time. GFB probably did not induce resistance in cells but lowered the death rate by exerting a protective effect. However, this effect was quite different from the protection exerted by some prebiotics. For example, fructooligosaccharides and inulin induced a prolonged tail as a result of a general stress response mechanism due to starvation [34,35], whereas GFB resulted in a reduction of the death kinetics but not a tail effect. The lowering of death rate in the bacterial curve, as well as growth enhancement, is generally the result of a protective compound and has observed previously in some cell-free filtrates or bifidogenic factors. The effects of these factors were variable and described as growth enhancement due to membrane permeability, combating cell aging, etc. [36,37].

The high temperature generated by the microwave treatment and applied to the hydrated wheat caryopses for a short period of time to detoxify gluten may break the hydrogen bonds between the glutamine residues in proteins when in their native form in protein bodies, inducing a rearrangement of the secondary and tertiary structure of the gluten protein, with a different spatial conformation of the toxic sequences [16]. We also postulated that the rearrangement of some of the gluten protein

structure involves the exposure of positive charges [18]. Positive molecules, namely cationic peptides, can exert a strong antibacterial effect because they supposedly act at the cytoplasmic membrane, leading to permeabilisation and eventually membrane disruption. Arginine and lysine residues play a major role in this process [38].

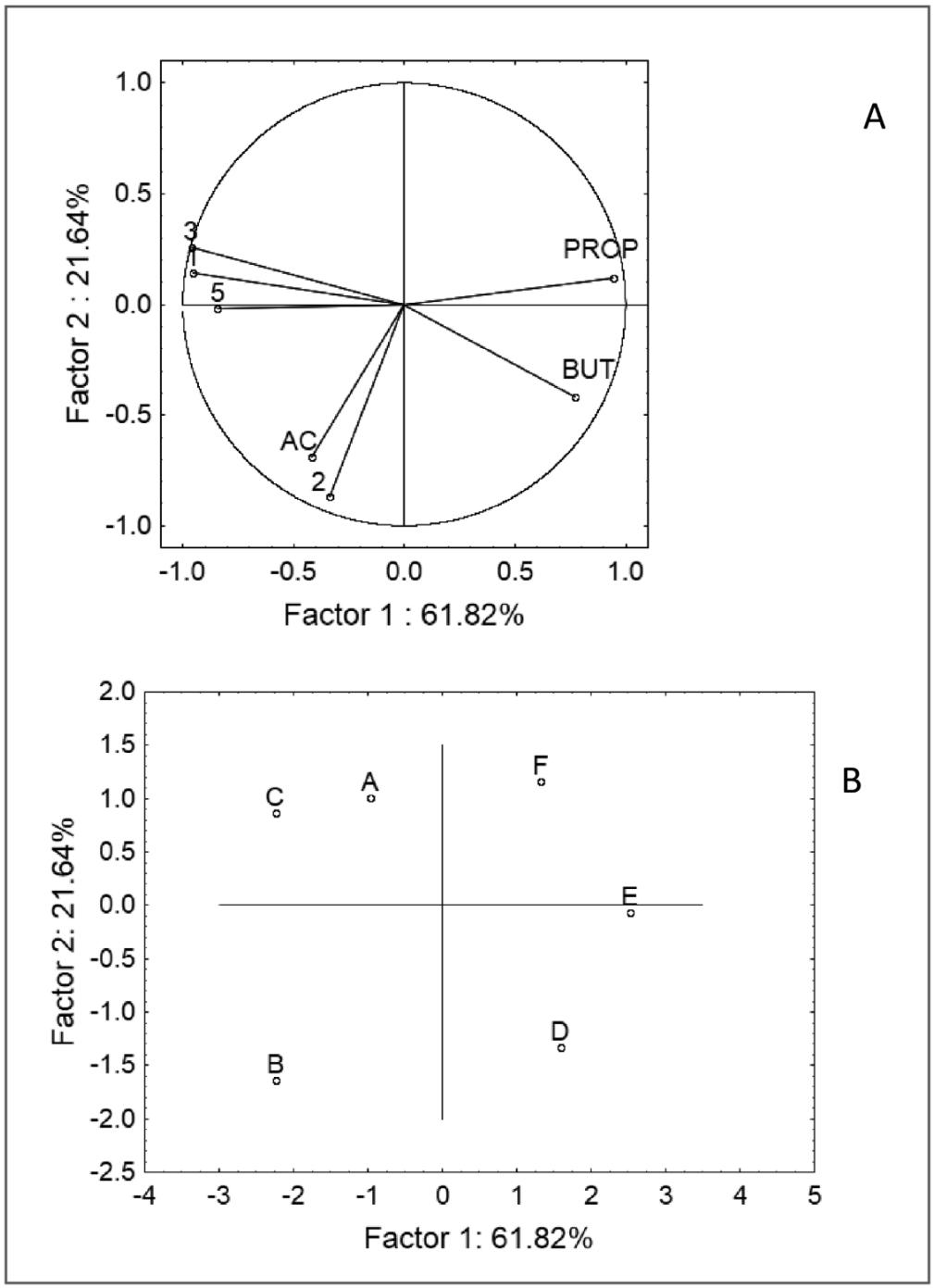


Fig 5. PCA based on the SCFA and FISH data after 24 h of fermentation in pH-controlled batch cultures. A) Variable projection; B) Case projection.
doi:10.1371/journal.pone.0162770.g005

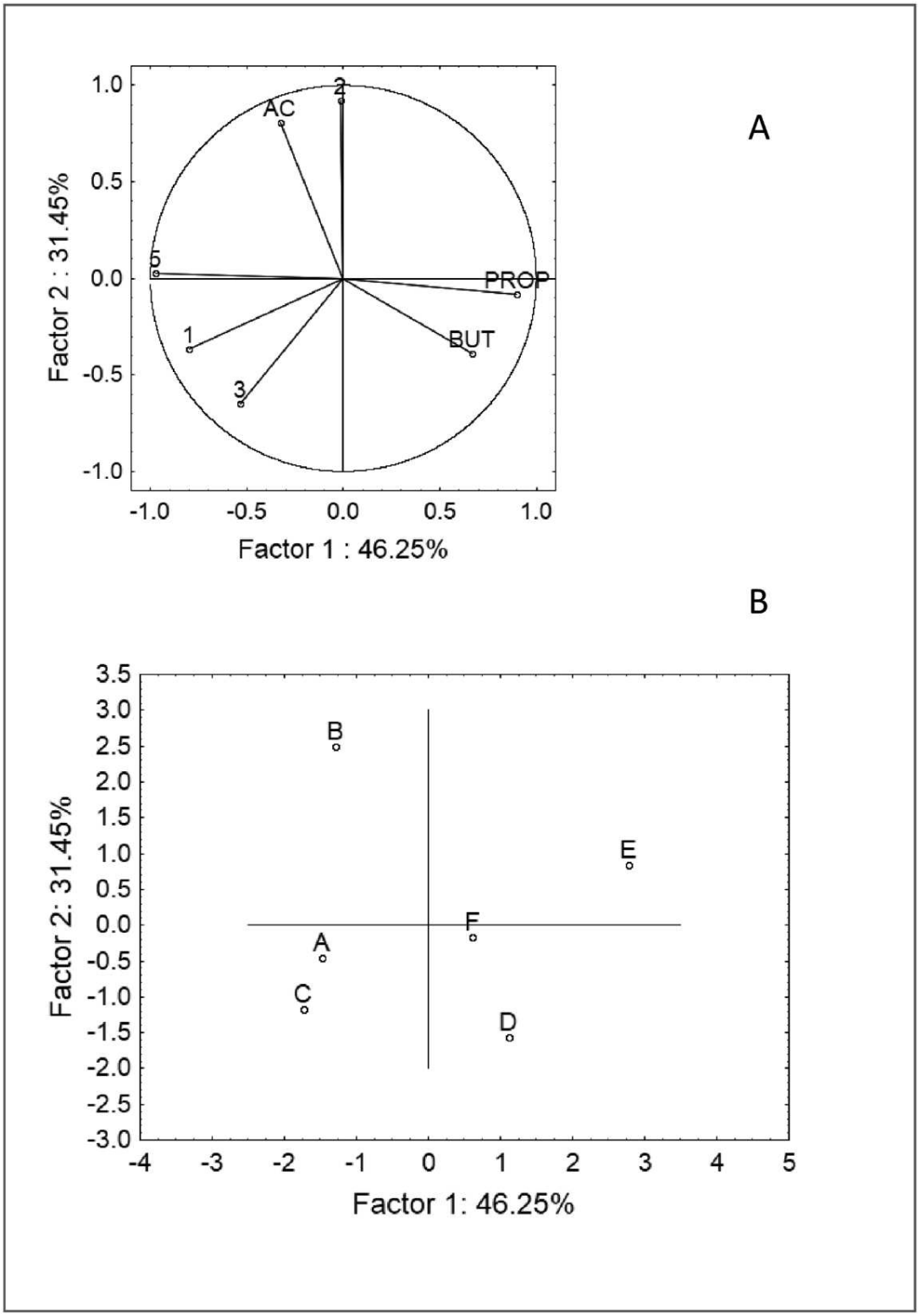


Fig 6. PCA based on the SCFA and FISH data after 48 h of fermentation in pH-controlled batch cultures. A) Variable projection; B) Case projection.
doi:10.1371/journal.pone.0162770.g006

Moreover, the interaction of antimicrobial peptides with anionic membrane phospholipids is a key factor in killing bacteria [38]. To date, it is not clear whether the outer membrane of Gram-negative

To date, it is not clear whether the outer membrane of Gram-negative bacteria can exert a positive or negative effect. Our results suggest that the outer membrane did not play a role due to the significant antibacterial effect on both Salmonella Typhimurium and S. aureus.bacteria can exert a positive or negative effect.

Teichoic acid can act negatively on cationic peptides because they have a negative charge, catch positive molecules and decrease their potential towards cells [38]. This idea could partially explain how GFB did not exert a negative effect on lactobacilli and bifidobacteria but did for S. aureus, and this difference might be the result of the unique structure of the cell wall and teichoic acids in lactobacilli, as described by Chapot-Chartier and Kulakauskas [39].

After this preliminary evaluation in strictly controlled conditions, we moved to a complex ecosystem to assess whether GFB can affect the evolution of heterogeneous microbiota. Two variables were assessed: the type of bread (CB and GFB) and the subject (coeliac or healthy people). The experiments were performed using 6 different subjects, and a negative control (batch culture inoculated with faecal microbiota but not supplemented with any type of bread) was also added for each subject. In addition, we also assumed that the faecal microbiota could experience a qualitative-quantitative change per se (decrease or increase without supplementation due to a "donor effect") or after bread supplementation (change due a "bread effect"). Thus, we used a static approach to standardize the data to the negative control at each sampling time. For each group (healthy subjects, A-C; coeliac subjects, D-F), the standardized values of the negative controls (A and D) showed the donor effect not related to bread supplementation. Therefore, a negative value pinpointed that bread supplementation caused a decrease in the viable count, whereas a positive value highlighted an increase.

Some groups were chosen as tests to assess the effect of CB and GFB, such as lactobacilli, bifidobacteria, Bacteroidetes, eubacteria and clostridia. Bifidobacteria can produce vitamins (e.g., K, B12, biotin, folate, thiamine) [31]. The synthesis of secondary bile acids is mediated via Lactobacillus spp. and Bifidobacterium spp. [31]. Moreover, Bifidobacterium spp. can also help prevent pathogenic infection through the production of acetate [40]. To date, the role of Bacteroides is controversial: some authors have postulated a positive impact, whereas other researchers have found a strong correlation of these microorganisms with CD [41,42] and a possible role in the pro-inflammatory response [42], mucin degradation and increased permeability of the small intestine [43,44]. E. rectale (now reclassified as Agathobacter rectalis) [45] is generally related to bifidobacteria, as it produces butyrate from acetate [46], with a beneficial effect on the host. The most valuable results were found for lactobacilli and bifidobacteria. In fact, the standardised values of lactobacilli from coeliac donors after 6 h were negative, suggesting that the lactobacilli population suffered a type of stress that was

enhanced by bread supplementation. The shift from negative to positive values in coeliac subjects in the presence of GFB (sample F) suggests that the supplementation suddenly interrupted this stress and beneficially modulated the microbiota composition. However, this effect was reversible because the samples experienced a partial shift after 48 h, suggesting that a prolonged and a beneficial effect could also be the result of prolonged supplementation. The experimental data from the batch cultures also confirmed the ability of GFB to promote the growth of Lactobacillus spp. The shift was much stronger in coeliac donors probably due the unbalanced microbiota composition compared to healthy subjects.

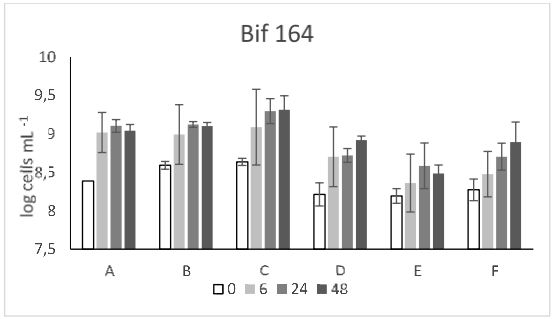
Differently from the screening, GFB exerted a positive effect on the bifidobacteria numbers of coeliac donors, although the effect was found after only 48 h. This result, along with the death kinetics data in saline solution, suggests that bifidobacteria probably require prolonged supplementation.

The same approach was used to model and analyse SCFA profiles. There is growing recognition of the role of SCFAs in immune function and inflammation in tissues [47]. Moreover, SCFAs can act as key sources of energy for colorectal tissues and bacteria and promote cellular mechanisms that maintain tissue integrity [48–50]. The data were quite variable, and bread supplementation did not exert a clear effect. To better understand this scenario, we decided to combine the SCFA data with the viable counts and pinpoint the changes at a global level.

Thus, the last statistical analysis (PCA) pinpointed a change in the ecosystem, and this effect was clearly distinguishable after 24 hours for sample F (coeliac donor+GFB), which moved from the region of coeliac people to healthy subjects. Thus suggests that GFB can cause a change in the whole ecosystem and exert a key role in the fight against the dysbiosis in coeliac people. This study provides new insights into the role of GFB on the qualitative-quantitative modulation of microbiota in simple or complex systems. The first step pinpointed that GFB has an important role in the prolongation of the survival of L. acidophilus and the antibacterial effect towards S. aureus and Salmonella Typhimurium. In a complex ecosystem, such as gut microbiota, GFB induced a beneficial modulation in terms of bifidogenic effects and on the growth of lactobacilli. Moreover, a final multivariate approach combining both the viable count and SCFA profile suggested that GFB causes a shift in the whole ecosystem. Therefore, this paper provides findings supporting the utilization of GFB to modulate the composition and metabolic profile of the intestinal microbiota in coeliac individuals. The applicability of such changes remains to be shown in a 3-stage continuous in vitro colonic model and an in vivo trial.

Supporting Information

A



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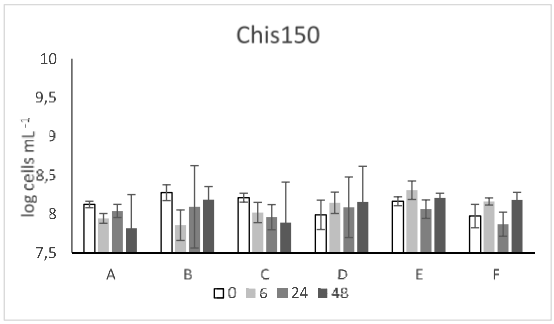

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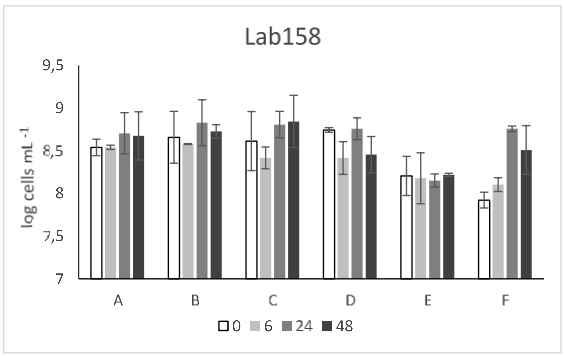
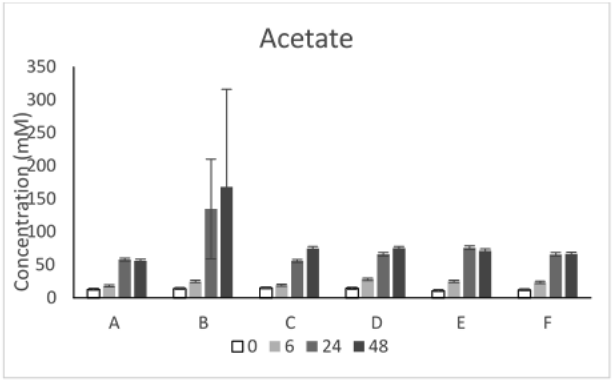


S1 Fig. Cell counts (mean±SD) of Bifidobacterium spp. (Bif164), Clostridium hystoliticum/perfringens group (Chis150) and lactobacilli/enterococci (Lab158) in pH-controlled batch systems at the beginning and after 6, 24 and 48 h of fermentation. A, negative control healthy donors; B, healthy donors + control bread; C, healthy donors + gluten friendly bread; D, negative control coeliac donors; E, coeliac donors + control bread; F, coeliac donors + gluten friendly bread. The data were preliminary analyzed to exclude the outliers.

A



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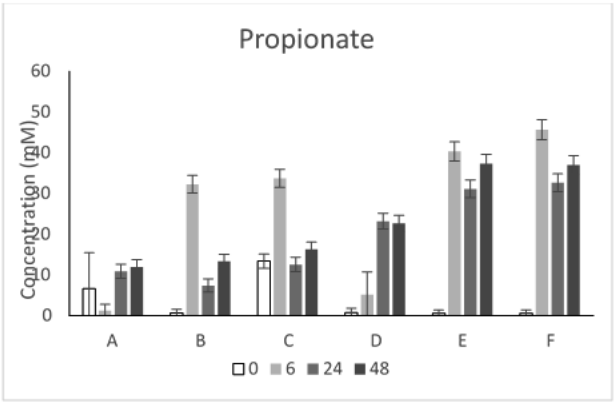

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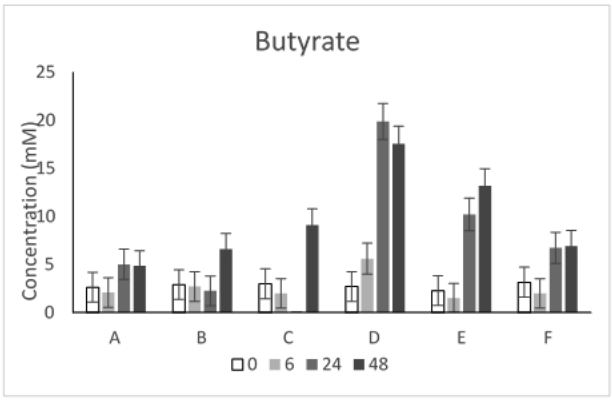

S2 Fig. Concentration (mM) of acetate, propionate and butyrate in pH-controlled batch systems at the beginning and after 6, 24 and 48 h of fermentation. A, negative control healthy donors; B, healthy donors + control bread; C, healthy donors + gluten friendly bread; D, negative control coeliac donors; E, coeliac donors + control bread; F, coeliac donors + gluten friendly bread. The data were preliminary analyzed to exclude the outliers.

Sample	log N_0*	δ	p	R
CB 0.4 g L^{-1}	8.09±0.14	16.98±0.90	1.42±0.11	0.991
GFB 0.4 g L^{-1}	8.18±0.13	17.03±2.06	1.44±0.14	0.999
CB 0.8 g L^{-1}	8.29±0.12	17.23±2.00	1.34±0.08	0.992
GFB 0.8 g L^{-1}	8.06±0.14	17.27±0.35	1.25±0.17	0.995

*log N_0, initial cell count (log CFU mL^{-1}); δ, first reduction time (h); p, shape parameter.

S1 Table. Fitting parameters of the Weibull equation for the death kinetics of B. animalis subsp. lactis (mean values ± SE). CB, control bread; GFB, gluten-friendly bread.

	L. acidophilus	*B. animalis* subsp. *lactis*
Inoculum	8.07±0.02A	8.70±0.07A
0.8 g L^{-1}		
CB	7.14±0.17B	8.54±0.05A
GFB	7.40±0.11B	8.51±0.07A
5.0 g L^{-1}		
CB	7.57±0.14A,B	9.30±0.04B
GFB	7.73±0.07 A,B	9.25±0.03B

S2 Table. Cell counts of L. acidophilus and B. animalis subsp. lactis (mean±SD) (log CFU mL-1) in a saline solution supplemented with either control (CB) or gluten-friendly bread (GFB) (0.8 and 5.0 g L-1) (incubation at 37°C for 24 h). The letters indicate the significant differences within each column (one-way ANOVA and Tukey's test, $P<0.05$).

		Homogeneous groups			
Sample	**FISH (log cells mL^{-1})**	**I**	**II**	**III**	**IV**
6 h					
A	-0.09				
D	-0.04				
E	0.08				
B	0.11				
C	0.13				
F	0.18				
24 h					
D	-0.19				
E	-0.17				
F	0.03				
A	0.41				
B	0.43				
C	0.64				
48 h					
B	-0.33				
F	-0.31				
E	-0.31				
D	-0.19				
C	-0.11				
A	-0.03				

S3 Table. One-way ANOVA ($P<0.05$) and homogeneous groups based on FISH data for Bacteroides/Prevotella group (enumerated as Bac303) after 6, 24 and 48 h of fermentation in pH-controlled batch culture systems. Increases/decreases refer to the inoculum of the negative control. Samples: A, negative control healthy donors; B, healthy donors + CB; C, healthy donors + GFB; D, negative control coeliac donors; E, coeliac donors + CB; F, coeliac donors + GFB. CB, control bread; GFB, gluten-friendly bread.

		Homogeneous groups		
Sample	FISH (log cells mL^{-1})	I	II	III
6 h				
B	0.00			
A	0.09			
C	0.13			
E	0.16			
D	0.37			
F	0.38			
24 h				
F	-0.06			
A	0.08			
E	0.17			
C	0.29			
D	0.44			
B	0.48			
48 h				
D	-0.02			
C	0.05			
F	0.06			
A	0.15			
E	0.22			
B	0.27			

S4 Table. One-way ANOVA ($P<0.05$) and homogeneous groups based on FISH data for E. rectale/C. coccoides group (enumerated as Erec482) after 6, 24 and 48 h of fermentation in pH-controlled batch culture systems. Increases/decreases refer to the inoculum of the negative control. Samples: A, negative control healthy donors; B, healthy donors + CB; C, healthy donors + GFB; D, negative control coeliac donors; E, coeliac donors + CB; F, coeliac donors + GFB. CB, control bread; GFB, gluten-friendly bread.

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Chapter 6

In Vitro Fermentation of Gluten Friendly Bread in Healthy human and celiac subjects: impact on gut microbiota

(In preparation)

Abstract

Patients with coeliac disease (CD) have altered intestinal microbiota compared to healthy individuals. Generally, gluten-free diet alleviates many of the symptoms, but somewhat surprisingly, it does not completely restore healthy microbiota profiles. Thus, the long-term result may be weaker immune defences and chronic inflammation.

Recently, a new and innovative gluten detoxification method has been developed (PCT/IB2013/000797). It is usually referred to as "gluten friendly" (GF) and lies in the application of microwave energy for few seconds to hydrated wheat kernels. This technology induces structural modifications to endosperm components which abolish the antigenic capacity of gluten and dramatically reduce, *in vitro*, the immunogenicity of the most common epitopes involved in CD, without compromising both the nutritional and technological properties necessary to process flour into bread, pasta and other baked goods. This study aimed to investigate the *in vitro* effects of GF bread (GFB) and control bread on the intestinal microbiota and metabolites composition of healthy and coeliac individuals. A validated three-stage continuous fermentative system simulating the proximal, transverse and distal parts of human colon (vessel 1, 2 and 3, respectively) was used to resemble the complexity and diversity of the intestinal microbiota. Main bacterial groups of the faecal microbiota were evaluated using 16S rRNA-based fluorescence *in situ* hybridization (FISH). Potential effects on microbial metabolism were also studied measuring short chain fatty acids (SCFAs) by HPLC analysis.

GFB showed positive modulations of the microbiota composition, as well as increases in SCFA concentrations in healthy and coeliac donors. Following GFB fermentation, beneficial modulations were seen in terms of bifidogenic effects in healthy and coeliac donors, as well as the growth in *Clostridium* clusters XIVa+b numbers in coeliac subjects. In healthy donors, acetic and propionic acid concentrations decreased in V1 from healthy individuals, whereas the levels of acetic increased in V2 and V3, and butyric in V3. Moreover, in case of coeliac individuals, we found significant increased levels of acetic and propionic in V1, as well as propionic in V2.

This study provides encouraging findings supporting the utilization of GF products with a positive effect on the intestinal microbiota and metabolites composition in coeliac people. However, further *in vivo* investigations are needed. Furthermore, High-Performance Computing techniques are ongoing to better understand how the 3-dimensional structures of the gluten proteins at the molecular level affects their functional characteristics.

Introduction

To date, it has been well-established that the composition and metabolism of human microbiota play crucial roles in human health. Microbial colonization of the gastrointestinal tract varies widely, with the large intestine having not only the highest density of microbes in terms of bacterial cells per gram but also the most metabolically active microbial community (Turnbaugh et al., 2006). Genetics, mode of birth, infant feeding patterns, antibiotic usage, sanitary living conditions and long term dietary habits contribute to shaping the composition of the gut microbiome. Diet clearly has a major impact on variation in the gut microbiota composition, which is easily detected in faecal samples even after only a few days after a change in diet (Muegge et al., 2011). The fermentative but also (anaerobic) respiratory bacterial metabolism of dietary components produces an extraordinary chemical diversity in the large intestine with protective (e.g., SCFAs) or detrimental (e.g., hydrogen sulfite, phenol, p-cresol or bile acids) effects on disease development (Flint et al., 2012). There have been numerous attempts to identify a "core" microbiota, usually defined as bacterial taxa that are shared between 95% of individuals tested. Identification of a core microbiome is important for defining a "normal" healthy state from which major variations may indicate a dysbiotic system that can result from or contribute to disease development. One barrier to define an intestinal core microbiome has been the vast degree of variation between individuals. The microbial communities identified in samples collected from an individual over time are more similar to each other than microbial communities between two individuals, although related persons share more bacterial strains than unrelated individuals. Although a consensus for what constitutes a core gut microbiome has been elusive, one report suggested that an international cohort of 39 individuals could be assigned to one of three distinct clusters or "enterotypes" based on metagenomic sequences. Arumugam et al. (2011) have found that each cluster was dominated by a particular bacterial genus (*Bacteroides, Prevotella*, and *Ruminococcus*) with positive or negative associations with a number of other genera in the community. They also reported that each cluster was enriched for specific gene functions that reflected different microbial trophic chains. The foodborne microbes transiently colonized the gut, introducing the idea that food may not only select for commensal bacterial species, but serve as a reservoir for new microbial introductions. Intentional introduction of prebiotic food ingredients and foods high in fibre can also be a means of subtly changing the relative abundance of bacterial species in the gut (Preidis et al., 2009). It is well-established that end products of carbohydrate metabolism can be positive for health. In this context, prebiotics are resistant to digestion and can become available for bacteria in the colon to produce SCFAs and inhibit the production of harmful metabolites. Thus, despite the inherent stability of the microbiome over time, changes related to weight loss and diet composition continue to subtly alter the composition and relative abundance of

our commensal organisms, driving the development of our gut microbiome throughout adulthood. To date, all different gastrointestinal diseases have evidence for a role of gut microbiota.

In particular, recent reports have shown how changes in the intestinal microbiome in coeliac patients might be altered and influenced underlying mucosal immune response. Coeliac disease is a chronic immune-mediated enteropathy triggered by the ingestion of gluten, the water-insoluble protein fraction in wheat, rye and barley, in patients who are HLA-DQ2 or HLA-DQ8 positive. The role of wheat gluten proteins in triggering CD is well-established, with gliadin and glutenin proteins being the major cause. Currently, 31 short peptide sequences in wheat gluten proteins, and related proteins in barley and rye, have been defined as being coeliac toxic: these are often referred to as coeliac 'epitopes'. Nowadays, the prevalence of CD in Europe and countries with high proportions of populations of European ancestry (e.g. the US, Australia) is now widely estimated as about 1% of the population, although substantial variation occurs between countries, from as low as 0.2% to over 5%. Within Europe, Finland has a particularly high incidence, reported as 1–2.4% (Maki et al. 2003; Godfrey et al. 2010; Mustalahti et al. 2010; Walker et al. 2011; Rubio-Tapia et al. 2012). There is a perception that the prevalence of CD is increasing, although this may be the result, at least in part, of increased awareness and improved diagnosis. Patients with CD have altered intestinal microbiota compared to healthy individuals. Generally, gluten-free diet alleviates many of the symptoms, but somewhat surprisingly, it does not completely restore healthy microbiota profiles. Thus, the long-term result may be weaker immune defenses and chronic inflammation. Recently, a new and innovative gluten detoxification method has been developed (PCT/IB2013/000797) (Lamacchia et al., 2013; 2015a). It is usually referred to as "gluten friendly" (GF) and lies in the application of microwave energy for few seconds to hydrated wheat kernels. This technology induces structural modifications to endosperm components which abolish the antigenic capacity of gluten and dramatically reduce, *in vitro*, the immunogenicity of the most common epitopes involved in CD (Lamacchia et al., 2015b; 2016), without compromising both the nutritional and technological properties necessary to process flour into bread, pasta and other baked goods.

This study aimed to investigate the impact of the administration of GF bread (GFB) on the faecal microbiota and metabolism of healthy and coeliac volunteers using a three-stage continuous culture colonic model system. This is a useful tool to monitor the ecology and metabolic activities of the microbiota in the proximal, transverse and distal colon, in particular in relation to different environmental conditions, dietary intervention and the administration of drugs and antimicrobials. The influence of a control (without detoxification method) on the intestinal microbial ecology and bacterial metabolic profiles was also assessed

Materials and Methods

Substrate

The GFB and control bread were supplied by Molino Casillo S.p.A. (http://www.casillogroup.it/en/home-eng.html). Flour treated with microwave was called Gluten Friendly Flour (GFF) and was obtained by milling the microwave-treated caryopses (Italian priority patent n° 102015000084813 filed on 17.12.15, Methods for the detoxification of gluten proteins from grains of cereals and related medical uses. Inventor: Lamacchia C.). 100 g of cleaned wheat grains were dampened until reaching 15-18% humidity, measured with a halogen thermal balance (Mettler Toledo, HB43-S, Swiss), and subjected to rapid heating with microwaves (Delonghi, Italy, about 1 min. between 1000 and 750 watt) and slow evaporation of water. The rapid heating process and the slow evaporation of water was repeated until reaching a final temperature of 80-90°C, measured with a thermal camera (FLUKE i 20 model, Italy), and a moisture degree of 13-13.5% of 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 a roller mill. Flour coming from the milling of the not microwave-treated caryopses was called Control Flour (CF). The particle size of the GFF and the CF used was in the range of 100 to 200 □m.

Bread production

Bread was produced by using either control (CB) or gluten friendly flour (GFB) accordingly to the Chorleywood Breadmaking Process (CBP). Bread was prepared as follows: flour, 100 g; water, 66 mL; yeast, 1.33 g; salt, 1 g.

Simulated *in vitro* human digestion

Prior to being added to the batch culture systems, the two different types of bread (control and GF) were digested *in vitro*, under appropriate conditions according to the procedures described by Maccaferri et al. (2012). The selected breads (60 g) were mixed with 150 ml of sterile distilled water and homogenised in a stomacher (Seward, Worthing, U.K), at high speed for 5 min. α-Amylase (20 mg) was mixed with 1 mM CaCl2 (6.25 ml, pH 7.0) and added to the GFB solution, then incubated at 37 °C for 30 min, with shaking. After incubation, pH was adjusted to 2.0 and pepsin (2.7 g) in 0.1 M HCl (25 ml) was added, prior to a further incubation cycle, under shaking conditions, at 37 °C for 2 h. Finally, bile (3.5 g) and pancreatin (560 mg) were mixed with 0.5 M NaHCO3 (125 ml), the pH was adjusted to 7.0, and the mixture was incubated at 37 °C for 3 h. After this period, the sample solutions were stored at -80 °C.

Faecal inoculation

Faecal samples were obtained from two healthy subjects and two coeliac donors (male and female aged 30-50 years-old) who were free of any metabolic and gastrointestinal diseases, were not taking probiotic or prebiotic supplements, and had not taken antibiotics 6 months before faecal sample donation. This study was approved by The University of Roehampton research Ethics Committee (UREC 15/20). Faecal samples were placed in an anaerobic jar (AnaeroJarTM 2.5 L, Oxoid Ltd) including a gas-generating kit (AnaeroGenTM, Oxoid) in order to reproduce anaerobic conditions inside the chamber. An aliquot of 20 g of samples were diluted in 100 ml anaerobic PBS (0.1 mol/L phosphate buffer solution, pH 7.4, w/w) and homogenized (Stomacher 400, Seward, West Sussex, UK) for 2 minutes at 240 paddle beats per minute. Samples were added to anaerobic fermenters within 15 minutes of voiding.

Three-stage continuous culture gut model system

Physicochemical conditions in the colon were replicated in a three-stage continuous system, comprised of a cascade of three glass fermenters of increasing working volume connected in series. A small scale version of the validated system by Macfarlane et al. (1998) has been used for the first time in this study, representing the proximal (V1, 80ml), transverse (V2, 100ml), and distal colon (V3, 120ml) inoculated with 20% (wt:v) fecal homogenate from healthy and coeliac volunteers in a growth medium. The growth medium contained the following ingredients: starch, 5 g/L; mucin, 4 g/L; casein, 3 g/L; peptone water, 5 g/L; tryptone water, 5 g/L; bile salts, 0.4 g/L; yeast exact, 4.5 g/L; $FeSO_4$, 0.005 g/L; NaCl, 4.5 g/L; KCl, 4.5 g/L; $KH_2\ PO_4$, 0.5 g/L; $MgSO_4x7H_2O$, 1.25 g/L; $CaCl_2\ x6H_2O$, 0.15 g/L; $NaHCO_3$, 1.5 g/L; Tween 80, 1 mL; hemin, 0.05 g/L; and cysteine HCl, 0.8 g/L. Following inoculation, the colonic model was run as a batch culture for 24 h in order to stabilise the bacterial populations prior to the initiation of medium flow. After 24 h (T0), the medium flow was initiated and the system ran for 8 full volume turnovers to allow for steady state to be achieved (SS1) (assessed through stabilisation of the SCFA profiles (+/-5%). Taking into account the operating volume (300 mL) and the retention time (48 h, flow rate 6.25 mL/h) of the colonic model system, the control bread or GFB (3.75 mL) was added daily into V1. The bread was added to the system for a further 8 volume turnovers upon which steady state 2 (SS2) was achieved. Aliquots of 4.5 mL were removed at SS1 and SS2.

Short chain fatty acids (SCFAs) analysis by HPLC

The production of acetic, propionic and butyric acids in the fermentations was determined by HPLC (Merck, NJ) equipped with RI detection. The column used was an ion-exclusion REZEX-ROA organic acid column (Phenomenex Inc., UK) and temperature maintained at 84 °C. Sulphuric acid in HPLC-grade H_2O (0.0025 mmol/L) was used as the eluent, and the flow rate was maintained at

0.5mL/min. Aliquots of 1 ml collected from each vessel in microcentrifuge tubes were centrifuged at 1136 x *g* for 10 minutes to remove all particulate matter. The supernatants were then filtered using 0.22 µm low protein binding Durapore polyvianylidene fluoride (PVDF) membranes (Millex; EMD Millipore, Billerica, MA, USA). 20 µl of each sample was injected with a run of 45 min. Peaks were integrated using the Atlas Lab managing software (Thermo Lab Systems, Mainz, Germany). Quantification of the samples was obtained through calibration curves of acetic, propionic and butyric acids in concentrations ranging between 12.5 and 100 mM.

In vitro enumeration of bacterial populations by FISH

FISH analysis was performed as described by Costabile et al. (2014). Briefly, aliquots (375 µL) of gut model samples were fixed in three volumes of ice-cold 4% (w/v) paraformaldehyde for 4 h at 4ºC. They were then centrifuged at 13,000 x *g* for 5 min and washed twice in 1 ml of sterile PBS. The cells were again pelleted by centrifugation and re-suspended in 150 µl of sterile PBS, to which 150 µl of ethanol was added. Samples were then vortexed and stored at -20ºC until used in hybridisations. For the hybridisations, 20 µl of each sample was pipetted onto Teflon and poly-L-lysine-coated, six-well (10 mm diameter each) slides. The samples were dried onto the slides at 46ºC for 15 min and afterwards dehydrated in an alcohol series (50%, 80% and 96%, 3min each). Ethanol was allowed to evaporate from the slides before the probes were applied to the samples. A probe/hybridisation buffer mixture (5 µl of a 50 ng/µl stock of probe plus 45 µl of hybridisation buffer) was applied to the surface of each well. Hybridisations were performed for 4 h in an ISO20 oven. For the washing step, slides were placed in 50 ml of wash buffer containing 20 µl of DAPI (50 ng/µl) for 15 min. They were then briefly washed (2-3 s) in ice-cold water and dried under a stream of compressed air. 5 µ of antifade reagent were added to each well and a cover slip applied. Slides were stored in the dark at 4 ºC (for a maximum of 3 days) until cells were counted under a Nikon E400 Eclipse microscope. DAPI slides were visualized with the aid of a DM 400 filter and probe slides with the aid of a DM 575 filter. Numbers of specific bacteria were determined using the following equation:

$$DF \times ACC \times 6732.42 \times 50 \times DF_{sample}$$

Where,

DF = dilution factor (300/375 = 0.8)
ACC = average cell count of 15 fields of view
DF_{sample} = dilution of sample used with a particular probe or stain (e.g. 100x for Bif164 counts)
6732.42 = area of the well divided by the area of the field of view
50 = factor that takes the cell count back to per millilitre of sample

All probes were commercially synthesised and 5'-labelled with the Cy3-fluorescent dyeas reported in **Table 1.**

Probe	Target bacterial group/species	Target sequence (5'-3')	Hybridisation/ Washing T (ºC)	References
Bif164	*Bifidobacterium spp.*	CATCCGGCATTACCACCC	50-50	Langendijk et al. (1995)
Erec482	*Clostridium* clusters XIVa+b	GCTTCTTAGTCARGTACCG	50-50	Franks et al. (1998)
Lab158	*Lactobacillus/Enterococcus* spp.	GGTATTAGCAYCTGTTTCCA	50-50	Harmsen et al. (1999)
Chis150	*Clostridium histolyticum* group clusters I, II	TTATGCGGTATTAATCTYCCTTT	50-50	Franks et al. (1998)
Bac303	*Bacteroides–Prevotella* group	CCAATGTGGGGGACCTT	46-48	Manz et al. (1996)
Eub338 I[a]	Most bacteria	GCTGCCTCCCGTAGGAGT	46-48	Daims et al. (1999)
Eub338 II[a]	Most bacteria	GCAGCCACCCGTAGGTGT	46-48	Daims et al. (1999)
Eub338 III[a]	Most bacteria	GCTGCCACCCGTAGGTGT	46-48	Daims et al. (1999)

[a]These probes were used in equimolar concentrations (50 ng/mL). Formamide (35%) was included in the hybridisation buffer.

Table 1. FISH oligonucleotide probes used in this study.

Statistical analysis

All bacterial counts and SCFAs data were analysed by paired t-test in order to assess the significance of results of single pairs of data, when the overall P value of the experiment was below the value of significance ($P<0.05$). It was assumed equal variances and considered a two-tailed distribution. Analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

Results

Bacterial enumeration

Changes in the bacterial composition in gut model systems were assessed by FISH analysis (**Fig. 1** and **Fig. 2**). The results from the control bread in healthy people showed significant decreases in numbers of *Lactobacillus/Enterococcus* spp. (detected by Lab158 probe) (V1 and V2), *Bacteroides-Prevotella* group (V2) (detected by Bac303 probe) and *Clostridium* clusters XIVa+b (V1) (detected

by Erec482 probe) (**Fig. 1**). Trends in decreases were also observed for total bacteria in all the stages of the colonic model, although no significant differences were observed. Therefore, control bread did not impact drastically on the modulation of the faecal microbiota.

The administration of GFB led to a significant increase in bifidobacteria (detected by Bif164 probe) in coeliac and also healthy volunteers. In particular, in coeliac subjects a significant increase in bifidobacteria was observed from 8.42 to 8.90 Log CFU/ml ($P<0.05$) in the second stage of the colonic model and from 8.60 to 9.20 Log CFU/ml ($P<0.05$) in vessels 3, respectively. In healthy volunteers, there was a significant increase in numbers of bifidobacteria in vessel 3, from 7.90 to 8.40 Log CFU/ml ($P<0.05$) (**Fig. 2**). Significant increases were also observed in numbers of *Clostridium* cluster population in all the vessels for coeliac volunteers, from 8.85 to 9.50 Log CFU/ml ($P<0.05$); from 9.1 to 9.60 Log CFU/ml ($P<0.01$ and from 9 to 9.50 Log CFU/ml ($P<0.05$), respectively. General trends in increase were detected for all the other bacteria groups and in all vessels in both coeliac and healthy volunteers. However, no significant differences were found.

Short chain fatty acids production

SCFAs were measured in the three different vessels of the colonic model systems, at SS1 and SS2 by HPLC (**Fig. 3** and **Fig. 4**). The administration of control bread induced significant decreases in acetate (V1 and V2) and in propionate (V1), whereas butyrate increased in all vessels (Fig. 3).

The fermentation of GFB in healthy donors led to a significant production of acetate from 28.80 to 22.10 mM ($P<0.01$) in V1, from 44.40 to 56.94 mM ($P<0.01$) in V2 and from 46.00 to 76.50 mM ($P<0.001$) in V3 respectively. Furthermore, a significant increase in propionate concentrations was observed from 70.46 to 89.81 mM ($P<0.05$) in V1, as well as butyrate from 40.35 to 77.09 mM ($P<0.05$) in V3. In coeliac volunteers, significant increases of propionate levels were found 1 from 45.10 to 69.20 mM ($P<0.01$) in vessel 1 and from 50.80 to 70.20 mM ($P<0.05$) in vessel 2, respectively. A significant increase in the concentration of acetate in vessel 1from 41.20 to 89.00 mM ($P<0.01$) was also showed (**Fig. 4**).

Discussion

The human colon contains a wide range of bacterial communities, distributed in hundreds of distinct species, and the balance among them plays an important role in health and disease (Holzapfel et al., 1998; Rigottier-Gois et al., 2003). Substrates that escape from human digestion can be used for beneficial bacteria growth. Carbohydrate fermentation is the chief energy source for the gut microbiota; however, as colonic carbohydrates are used up in the proximal colon, saccharolytic fermentation decreases as the concentration of substrate decreases. Microorganisms then switch to

other energy sources, e.g. proteins, amino acids, and the end-products of these fermentations include SCFA but also branched-chain fatty acids, amines, indoles, sulfides, and phenols (Tuohy et al., 2012).

In this study, stool samples were collected from coeliac and healthy volunteers, in order to better understand the differences in microbiota composition and metabolic activity. This is the first study that has investigated the influence of GFB on complex faecal microbiota and metabolic profile *in vitro.*

Our data have shown significant increases in bifidobacteria numbers in response to GFB administration in both healthy and coeliac subjects compared to the control bread. This effect could be related to the to the new detoxification method. Bifidobacteria are recognised as one of the most important bacterial groups associated with human health, providing beneficial effects in the large intestine (Gibson et al., 1994; Russel et al., 2011). The GFB fermentation also led to increases in *Bacteroides* spp. group and significant changes of *Clostridium* cluster XIVa group. Both groups may exert a detrimental effect on colon health due to an association with some metabolites. However, they also contain saccharolytic species which can produce large concentrations of beneficial SCFAs from sugars. SCFAs produced by gut microbiota in the colon have an important role. Butyrate is often associated as an energy source for the epithelial cells and acetate plays an important role in controlling inflammation and combatting pathogen invasion (Russel et al., 2013). In addition, SCFAs help to regulate sodium and water absorption, and can enhance absorption of calcium and other minerals as well as lower the colonic pH. GFB *in vitro* fermentation induced a modulation of the colonic microbiome with increased acetate and propionate levels, which was not observed with control bread in healthy donors. The best known metabolic pathway for acetate and propionate production from gut bacteria, involves the metabolism of polysaccharides. Acetate production occurs mainly through the fructose-6- phospahte phosphoketolase pathway by bifidobacteria, and a greater production of this acid could be related to the increase of the bacteria (Miller et al., 1996). According to Hosseini et al. (2011), propionate can be produced from fermentable carbohydrates by two pathways. The first involves decarboxylation of succinate by the presence of *Bacteroides fragilis* and *Propionibacterium* spp. groups, while the second one is the acrylate pathway, in which pyruvate is first reduced to lactate by lactate dehydrogenase by the presence of some clusters of *Clostridial* groups. An increase in bifidobacteria, *Bacteroide*s and *E. rectale* groups was indeed observed during GFB fermentation.

GFB showed positive modulations of the microbiota composition, as well as increases in SCFA concentrations in both healthy and coeliac donors. Following GFB fermentation, beneficial modulations were seen in terms of bifidogenic effects in healthy and coeliac donors, as well as growth in *Clostridium* clusters XIVa+b numbers in coeliac subjects. Although acetic and propionic acids

decreased in V1 from healthy individuals, increased levels of acetic were found in V2 and V3, and butyric in V3. Moreover, in case of coeliac individuals, we found increased levels of acetic and propionic in V1, as well as propionic in V2. This *in vitro* work provides encouraging findings supporting the utilization of GF products with a positive effect on the intestinal microbiota and metabolites composition in coeliac people. However, further work will be done to assess such changes in an *in vivo* human intervention study.

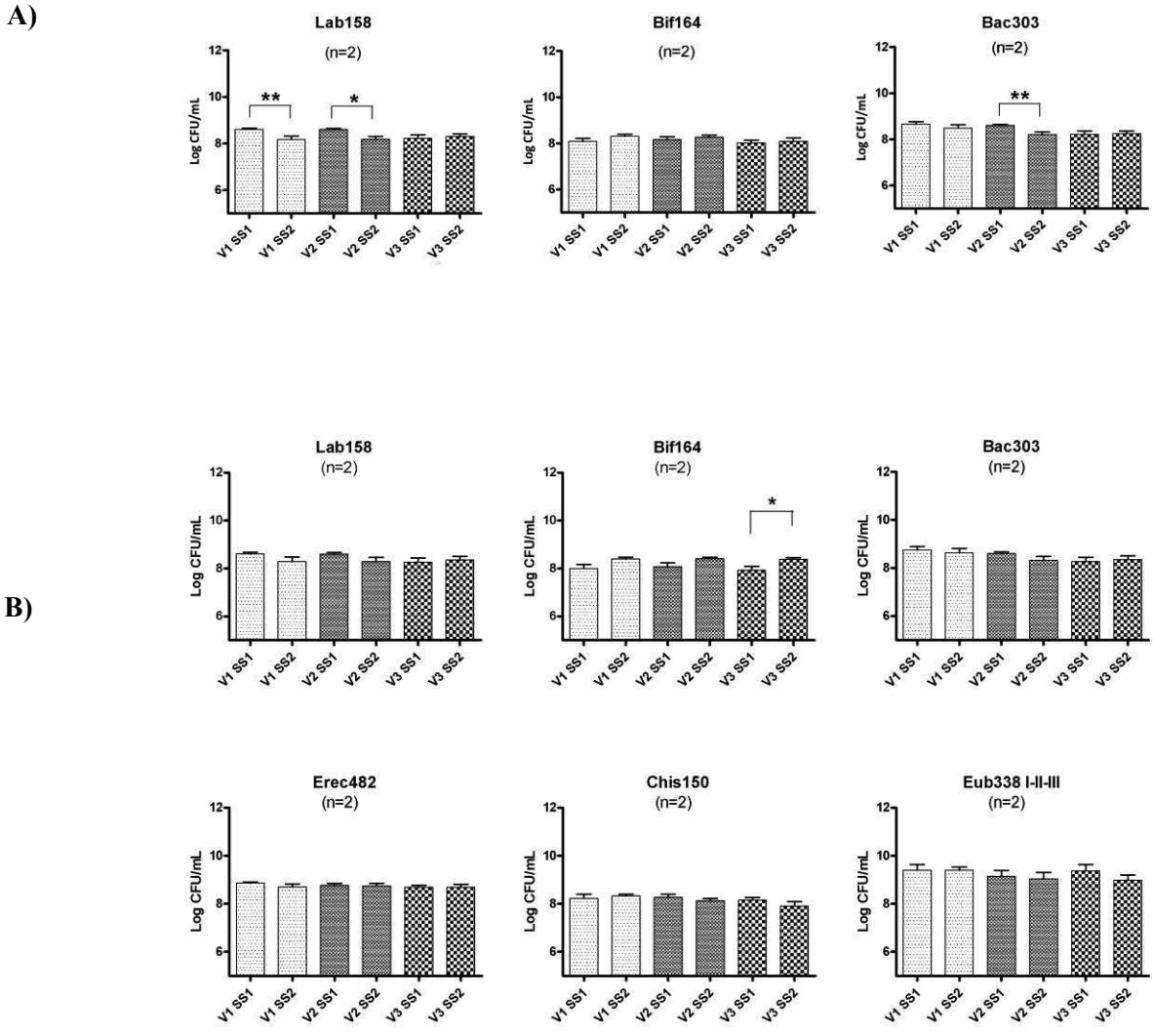
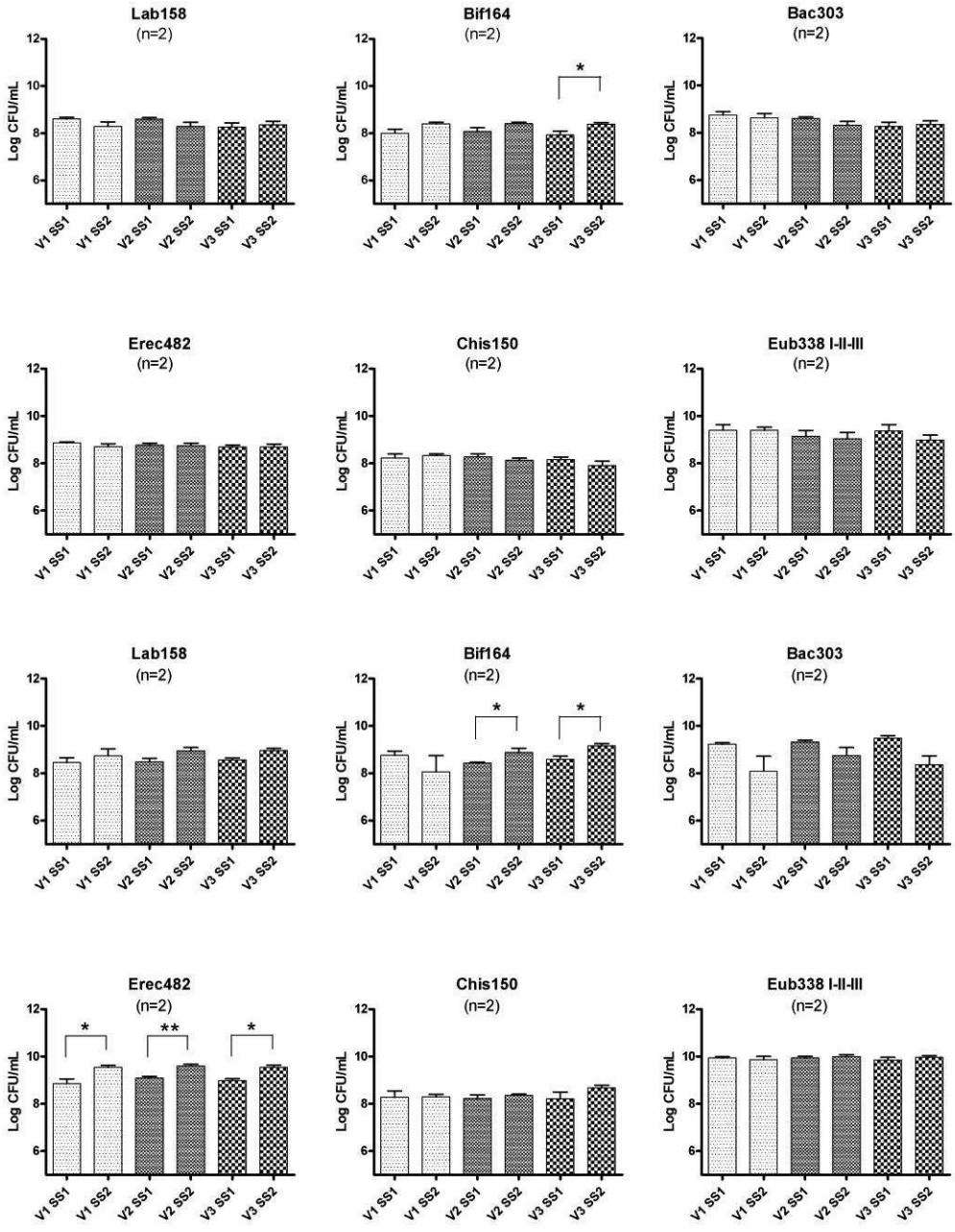


Figure 1. Bacterial groups in the culture broth recovered from the three different vessels (V1, V2 and V3) of the colonic model system before (SS1) and after (SS2) (A) control bread and (B) GFB in healthy donors. Results are reported as means of the data of two models±SEM (n=2).

A)



B)

Figure 2. Bacterial groups in the culture broth recovered from the three different vessels (V1, V2 and V3) of the colonic model system before (SS1) and after (SS2) GFB in (A) healthy and (B) coeliac donors. Results are reported as means of the data of two models±SEM (n=2).

A)

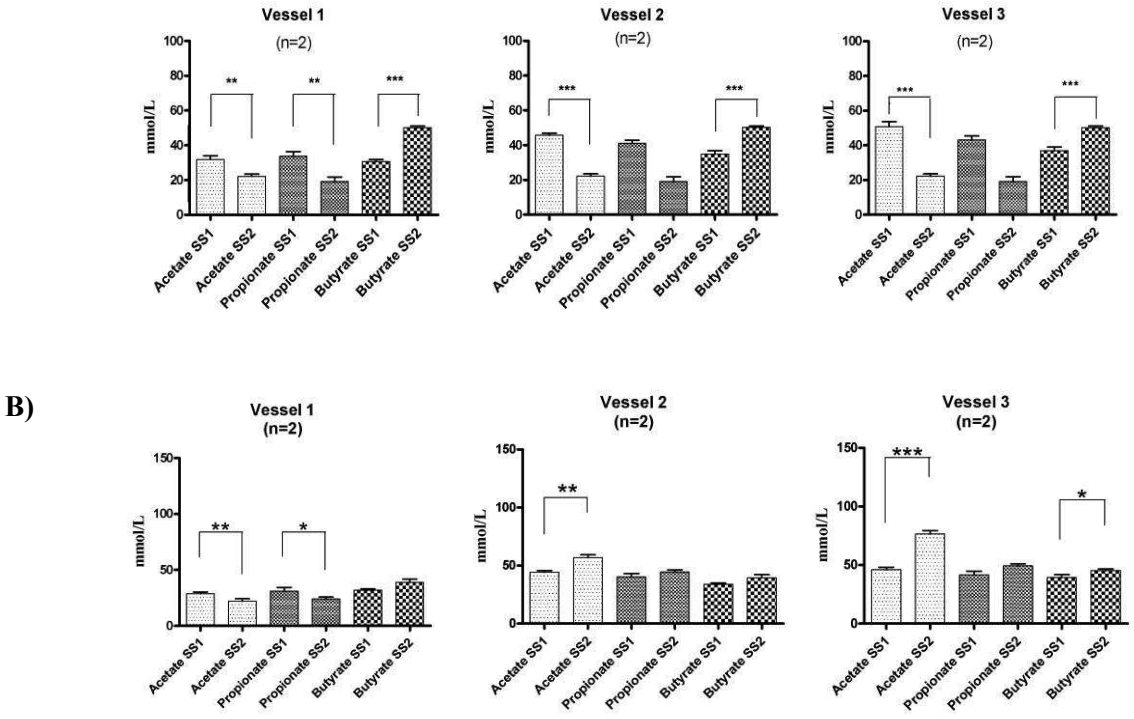


Figure 3. SCFA concentrations in the culture broths recovered from the three different vessels (V1, V2 and V3) of the colonic model system before (SS1) and after (SS2) (A) control bread and (B) GFB in healthy donors. Results are reported as means of the data of two models±SEM (n=2).

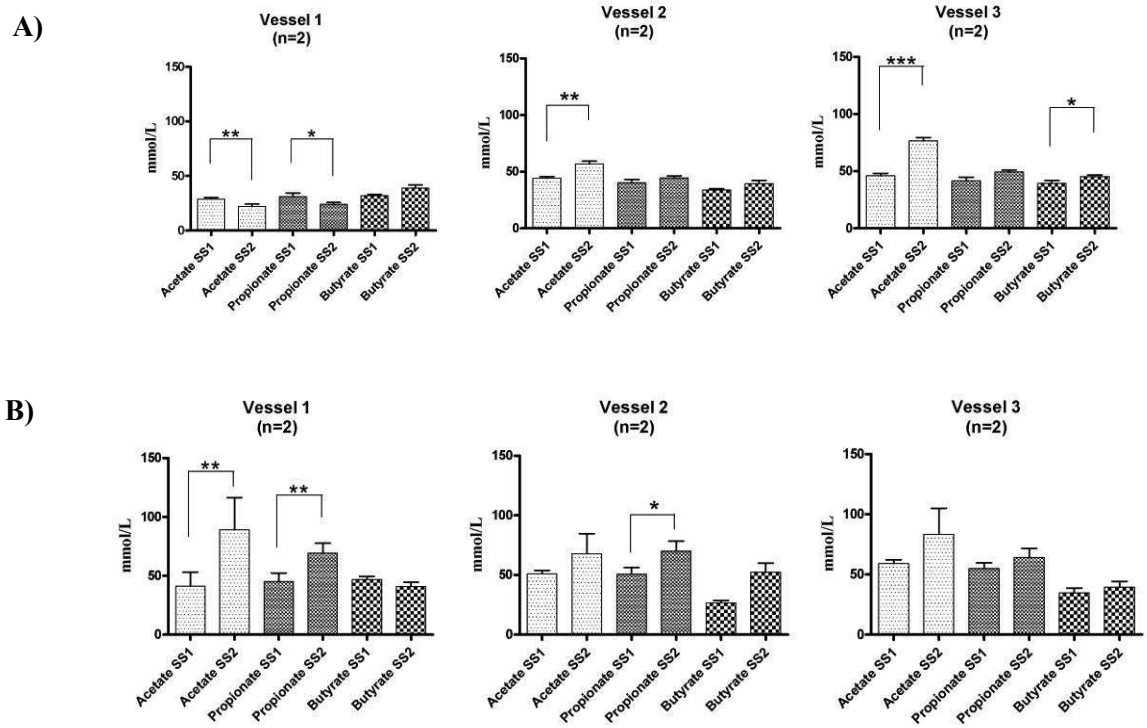


Figure 4. SCFA concentrations in the culture broths recovered from the three different vessels (V1, V2 and V3) of the colonic model system before (SS1) and after (SS2) GFB in (A) healthy and (B) coeliac donors. Results are reported as means of the data of two models±SEM (n=2)

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Chapter 7
General Discussion

The patented treatment used in this study and applied in hydrated wheat kernels reaching a high temperature for a short time induced significant changes in gluten proteins. Among these changes, the most important are the reduced antigenicity (by 99% in comparison with the control samples) and the solubility of gluten in saline aqueous solution. These changes suggest that treatment applied to wheat kernels may allow the breaking of hydrogen bonds between glutamine residues when proteins are in their native form in protein bodies, thus favouring protein conformational and/or structural changes. As proposed by the Codex Alimentarius Committee, the flour described in this study should be classified as a very low gluten content (21–100 ppm of gluten) for use in the production of food with superior sensory properties for the diet of patients with gluten-correlated disorders (intolerant and sensitive to gluten) or for those genetically predisposed to this pathology.

To better understand chemical changes in gluten proteins, *Gluten Friendly* flour were characterized by size exclusion high-performance liquid chromatography (SE-HPLC) and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

The SE-HPLC charts indicate that the treatment applied to mature wheat grains may not allow intermolecular bonding between proteins of different classes, leading to the formation of larger polymers and increasing the large polymer proteins peak. Protein profiles instead suggest the formation of intramolecular bonds into a single class of proteins that could be explained by the segregation of wheat proteins into different protein bodies (Lamacchia et al., 2010), which accumulate simultaneously and independently in wheat starchy endosperm cells, and by the qualitative and quantitative protein gradients in wheat endosperm. In fact, sub-aleurone cells are enriched in LMW-GS and gliadins, whereas HMW glutenins are only present in significant amounts in the inner part of the starchy endosperm (Tosi et al., 2011). However, the SE-HPLC chart of Gluten Friendly proteins does not show the aggregation of SMPs and their interaction with gliadins as seen by Lamacchia et al. (2010). This may be because the temperature applied in this study was significantly lower (110–120 °C) than that used in the previous study (Lamacchia et al., 2010) in which the seeds were arranged on a steel bearing placed into direct contact with a wood-fire for 120 s until the kernel browned. This is consistent with the observation that glutenins (LPP and SPP) are susceptible to an increase in temperature and that glutenin denaturation is complete at 60°C, whereas gliadins (LMP), because of their conformational structure, are more stable to the increase in temperature, and denaturation begins at 60 °C and increases significantly at 90 °C (Lamacchia et al., 2007; Schofield et al., 1983; Singh & MacRitchie, 2004). Furthermore the percentage of total UPP in the treated sample decreases significantly, consistent with the SE-HPLC chart and with the two area ratio values, but a concomitant increase in the S–S bonds is also detected. These results suggest that the treatment induces modification in the SH and disulphide bonds, moving polymeric proteins, and

in particular, the HMW (LPP) subunits towards larger insoluble protein aggregates, which become unextractable, even after sonication. This is consistent with the conclusion reached by Singh and MacRitchie (2004). The increase in the total cysteine, suggests that the treatment induces a major exposure of the cysteine residues due to the dissociation and unfolding of proteins. In fact, the modification of proteins by heat usually results in changes in the secondary and tertiary structure of the protein molecules that become denatured. The process of denaturation involves the dissociation and unfolding of proteins and is often accompanied by the formation of disulphide linkages and the exposure of hydrophobic amino acids on the surface. The degree of heat treatment results in differences in the extent of the denaturation and functionality of the proteins. The more the hydrophobic amino groups are exposed, the more insoluble the proteins become. To better understand this phenomenon, total proteins and the glutenin and gliadin fractions of CWF and TWF were separated by SDS–PAGE under reducing and non-reducing conditions. Analysis of these fractions by SDS–PAGE showed that the treatment did not cause either a decrease in the number of bands or a decrease in the intensity of the bands. These results, confirmed by quantification of band intensities obtained via image analysis, suggest that TWF proteins are not denatured, do not interact with other compounds in wheat endosperm and that the decrease of the total peak area observed by SE-HPLC analysis is primarily due to protein polymerization that occurs only through disulphide bonds. These last results are consistent with the work of Weegels et al. (1994a) and Weegels, de Groot, Verhoek, and Hamer (1994b) but are in contrast to the work of Guerrieri and Cerletti (1996). Guerrieri et al. (1996) and Lamacchia et al. (2010) suggest that, at temperatures higher than 100 °C, changes other than disulphide bridging are involved. This may be because heat-induced changes in the properties of gluten proteins may be a function, not only of the temperature reached, but also of both the heating time applied and the sample moisture content (Singh & MacRitchie, 2004; Weegels et al., 1994a,b). Because an increase in the number of cysteine residues was observed, we attempted to determine whether this treatment induced unfolding of the proteins with exposure of the amino acids to increase gluten antigenicity; so the amount of the potential celiac-toxic most repetitive pentapeptide epitope, glutamine–glutamine–proline–phenylalanine–proline (QQPFP), in gluten proteins was determined. In addition, control and treated flour gliadins samples were tested for their ability to bind a specific monoclonal antibody, R5, using the high sensitivity of the R5-ELISA (Valdés et al., 2003), which is used to monitor residual gluten levels as low as 3.2 ppm in all commercial gluten-free foods. Interestingly, the R5-ELISA indicated a significant reduction (99%) in the levels of detectable gluten protein, showing that with this microwave treatment, gliadin significantly decreases its cross-reactivity with the R5 monoclonal antibody. For Gluten Friendly flours, denaturation of the different protein classes occurs minimally and may be caused by their deposition in different protein bodies in

their native form, allowing for chemical changes otherwise impossible in the already formed gluten. This dramatically reduces the antigenicity of gluten. In addition the effect of treatment on gluten index (GI) of Gluten Friendly flours was also evaluated. Control and treated flour samples were analysed for GI and results showed that the isolation of gluten from the TWF samples was not possible. Consequently, it was also not possible to calculate a GI to determine its strength. These results suggest that the treatment induces significant modification of gluten proteins such that they become soluble in aqueous solution, although these modifications are not observed in the SDS–PAGE analysis. The main reasons for the water-insoluble characteristics of gluten are the hydrophobic nature of its proteins, consisting of a large percentage of uncharged amino acid residues (glutamines Gln; asparagines Asn) and the presence of hydrogen bonds. By transforming the amides of Gln and Asn into carboxyl groups through deamidation, the solubility of proteins in wheat gluten is transformed with effects such as changing of the charge density, increasing the electrostatic repulsion and breaking the hydrogen bonds (Liao et al., 2010; Riha, Izzo, Zhang, & Ho, 1996). In our case, the solubility of gluten proteins cannot be due to the deamidation of amino acids because no difference between the CWF and TWF proteins bands was detected during the SDS–PAGE separation. However, high temperature for short time, generated by the treatment applied to the caryopses, may break the hydrogen bonds between protein glutamine residues (Shewry, Popineau, Lafiandra, & Belton, 2001) when proteins are in their native form in protein bodies, allowing conformational and/or structural changes (Liao et al., 2010), as evidenced by the increase in the total cysteine levels. This would explain the significant decrease in gliadin cross-reactivity towards the R5 monoclonal antibody because it recognizes the repetitive pentapeptide QQPFP epitope. In addition, the impact of Gluten-Friendly™ (GF) technology (Italian priority patent n°102015000084813 filed on 17th December 2015) on wheat kernel endosperm morphology and gluten protein structure, using SEM, light and immunofluorescent microscopy was also evaluated.

Several samples of CG (control grains) and GFG (gluten-friendly grains) were cleaved transversally into two halves and prepared for CPD (Critical-Point Drying) and freeze-fracturing analysis, and SEM-analyzed at a high voltage of 20.0 kV.

Micrographs of GFG clearly demonstrate the presence of a thick, confluent protein matrix in prismatic endosperm cells, with complete embedding of the starch granules. These results are in accordance with previous works that suggest that under heat stress, the endosperm of the kernels might appear increasingly aggregated, with the starch granules embedded in the protein matrix and a dense cellular structure (Pyler, 1988, Dias & Lidon, 2009).

The effects of Gluten-Friendly™ technology on gluten protein structure was also evaluated by *SEM–Immunogold* and *Immunofluorescence* experiments with two monoclonal antibodies specific for

repeated sequences common to gliadin and LMW fractions. Results showed a significant ($p<0.01$) decrease in silver concentration in GFG seeds compared with CG, after labelling with the antibodies. Similarly, *Immunofluorescence* experiments in GFG samples showed a strong and significant decrease in signal intensity, observed after labelling with antibody 0610 and the antibody specific for γ-gliadin compared to the CG samples, in agreement with the *SEM* results.

These results confirm that Gluten-Friendly™ technology induces significant changes in gluten proteins, thus reducing cross-reactivity with antibodies that recognize almost the entire range of gluten proteins through the sequences QQSF, QQSY, PEQPFPQGC. Lamacchia et al. (2016) suggested that the brief high temperatures generated by microwaves applied to the hydrated caryopses lead to conformational and/or structural changes in the proteins, as evidenced by the increase in total cysteine levels. Furthermore, the analysis by SDS-PAGE of protein fractions under reducing and non-reducing conditions showed that microwave treatment causes neither a decrease in the number of the bands nor a decrease in their intensity (Lamacchia et al., 2016); thus, a rearrangement of the secondary and/or tertiary structure in gluten proteins with a different spatial conformation of the aminoacid sequences could be suggested. Enlargement of part of the micrographs highlighted another interesting feature, i.e. a strong merging of protein bodies in the CG and GFG samples.

Indeed, micrographs of the samples revealed that the coalescence of protein bodies in mature wheat grains was complete and resulted in a continuous proteinaceous matrix surrounding the starch granules. Nevertheless, it was possible to still distinguish protein bodies and highlight differences in the merging level of protein bodies in GFG with respect to CG samples. In the GFG samples, protein bodies (PB-type2) were completely merged in the protein matrix, which appeared more homogeneous and confluent, showing a higher level of aggregation when compared to CG samples (PB-type1). A multivariate approach (PCA) confirmed that PB-type 1 and PB-type 2 were negatively related. These results suggest that aggregation may be the primary cause for the reduced cross-reactivity of proteins, rather than a change in secondary and/or tertiary structure. In any event, these findings are in accordance with analyses performed with SEM using freeze-fracturing techniques and with the fact that gluten proteins are susceptible to heat treatment and polymerize during heating (Schofield, Bottomley, Timms, & Booth, 1983). Gluten protein behaviour when subjected to relatively high temperatures has been studied by a number of workers and indeed the incorporation of gliadin monomers in the glutenin network through covalent bonds has been highlighted (Singh & MacRitchie, 2004; Redl, Morel, Bonicel, Vergnes, & Guilbert, 1999). However, Lamacchia, Baiano, Lamparelli, La Notte & Di Luccia (2010) showed, for the first time, that the application of high temperature to wheat grains generated protein polymerization in which gliadins did not cross-link with glutenins. The explanation for this phenomenon was that, within the kernel, the gluten protein

network has not yet formed and gluten proteins are deposited in different protein bodies. Furthermore, Lamacchia et al., (2016) confirmed these results, showing that the application of a short burst of high temperature to mature hydrated wheat kernels using microwave energy leads to protein polymerization between proteins of the same class (i.e. gliadin-gliadin, glutenin-glutenin, albumin-albumin) and only through disulphide bonds (evidenced by the SE-HPLC chart and SDS-PAGE analysis). However, this does not seem to be in accordance with the findings from the SEM and immunofluorescent experiments where images show a strong aggregation among all wheat kernel proteins. One explanation for this could be that the strong aggregation between different classes of proteins in seeds is due to ionic and/or hydrophobic interactions, caused by a rearrangement of the secondary and/or tertiary structure of the gluten protein molecules during heating.

Such aggregation was not visible through SE-HPLC and SDS-PAGE (Lamacchia et al., 2016) because of the use of sodium dodecyl sulphate-denaturing conditions in the extraction buffer.

At least, the effect of Gluten-Friendly™ technology on gluten protein antigenicity was evaluated by *Light Microscopy* using the R5 monoclonal antibody which recognizes the most repetitive potential coeliac-toxic pentapeptide epitope, glutamine-glutamine-proline-phenylalanine-proline (QQPFP) in gluten proteins and the epitopes LQPFP, QLPYP and PQPFP in the sequence of the recently-described potent inducer of gut-derived human T-cell lines in coeliac patients, the A gliadin 33-mer peptide of residues 57–89 (LQ LQPFPQPQLPYPQPQLPYPQPQL–PYPQPQPFP) (Arentz-Hansen, Korner, Molberg, Quarsten, Vader, Kooy, 2000). The reduced gluten antigenicity in GFG samples observed with the R5 colorimetric assay was in accordance with the *SEM–Immunogold* and *Immunofluorescence* experiments and with Lamacchia et al. (2016) that showed a drastic reduction (99%) in the levels of detectable proteins, treated with Gluten-Friendly™ technology, tested for their ability to bind the specific monoclonal antibody R5 in a sandwich Elisa test. R5-Elisa includes an extraction procedure allowing aggregates of gliadins generated by the heat process to be solubilized (Valdés, García, Llorente, & Méndez, 2003). On this basis, a rearrangement of the secondary and tertiary structure in gluten proteins, with a different spatial conformation of the toxic sequences, induced by Gluten-Friendly™ technology, and not a strong aggregation among proteins, can explain the significant reduction in antigenicity in GFG samples and therefore a significant reduction in cross-reactivity of gluten proteins with their own antibodies. This is well supported by the fact that the availability of antigenic fragments in gluten depends on the secondary and tertiary structure of the protein as well as on the SS bonds that stabilize particular conformations of epitopes to bind with the antibody (Waga, 2004). To better understand the chemical changes in Gluten Friendly Flour, the chemical, rheological and pasting prorperties was investigated. Results showed that the gluten became soluble in aqueous solution and it was not possible to isolate it using the Glutomatic

apparatus. In addition, a decrease of dough elasticity was detected using an extensiograph, dough stability decreased from 5 to 2 min, whereas farinograph water absorption of Gluten Friendly Flour was higher (+10%) than control flours. Rosell et al., (2001) reported that the differences in water absorption are caused predominantly by the greater number of hydroxyl groups that exist, which allows for more water interaction through hydrogen bonding. Furthermore, remarkable differences in dough characteristics between CF and GFF samples were established. From the results obtained, the energy of the dough (dough strength) and the dough extensibility were decreased in GFF samples when compared to CF. The significantly decrease ($p< 0.05$) in the elasticity of the treated flour samples is consistent with the observation that breaking the hydrogen bonds between gluten proteins and conformational changes induced by microwave treatment in the kernel allow polymers in the flour to be hydrated but not to form a high amount of loop to train structures that are important for determining dough elasticity (Lamacchia et al., 2016). Furthermore pasting temperature of Gluten Friendly Flours was not affected by the treatment, and starch properties and functionality were also preserved.

These results are in accordance with previous research, indicating that starch granules did not differ in number and shape in Gluten Friendly Grain and Control Grain samples, moreover they were dispersed in the continuous protein matrix (Landriscina et al., 2017).

Although deep modifications of gluten proteins, Gluten Friendly Flours was able to form dough and leaven and produce bread. The sensory qualities of Gluten Friendly Bread did not differ significantly ($p<0.05$) from the control bread.

Next study aimed to investigate the efficacy and safety of the "Gluten Friendly™" technology evaluating the *in vitro* response of healthy and coeliac human faecal microbiota to gluten-friendly bread (GFB), (Bevilacqua et al., 2016). Thus, GFB and control bread (CB) were fermented with faecal microbiota in pH-controlled batch cultures. The effects on the major groups of microbiota were monitored over 48 h incubations by fluorescence *in situ* hybridisation. Short-chain fatty acids (SCFAs) were measured by high-performance liquid chromatography (HPLC).

Furthermore, the death kinetics of *Lactobacillus acidophilus*, *Bifidobacterium animalis* subsp. *Lactis*, *Staphilococcus aureus*, and *Salmonella* Typhimurium in a saline solution supplemented with GFB or CB were also assessed. Coeliac people possess altered gut microbiota; in addition, many researchers pinpointed that dysbiosis dramatically impacts the host physiology (Khrishnan et al., 2015). Firstly, we aimed to determine whether GFB modifies the qualitative-quantitative composition of gut microbiota via a two-step plan. The first phase relied on the evaluation of the death decay of selected strains and focused on the eventual change of shape or kinetics of the characteristic curve of each microorganism. For this step, the Weibull function pinpointed some interesting results. As an

additional tool, we used a slightly modified Weibull equation to evaluate death (or survival time). Supplementation with GFB affected neither the first reduction time nor the shape parameter of L. acidophilus but exerted a significant effect on the death time. GFB probably did not induce resistance in cells but lowered the death rate by exerting a protective effect. However, this effect was quite different from the protection exerted by some prebiotics. For example, fructooligosaccharides and inulin induced a prolonged tail as a result of a general stress response mechanism due to starvation (Altieri et al., 2011; Altieri et al, 2013), whereas GFB resulted in a reduction of the death kinetics but not a tail effect. The lowering of death rate in the bacterial curve, as well as growth enhancement, is generally the result of a protective compound and has observed previously in some cell-free filtrates or bifidogenic factors. The effects of these factors were variable and described as growth enhancement due to membrane permeability, combating cell aging, etc. (Oda et al., 2013; Kang et al., 2015). The high temperature generated by the microwave treatment and applied to the hydrated wheat caryopses for a short period of time to detoxify gluten may break the hydrogen bonds between the glutamine residues in proteins when in their native form in protein bodies, inducing a rearrangement of the secondary and tertiary structure of the gluten protein, with a different spatial conformation of the toxic sequences (Lamacchia et al., 2016). We also postulated that the rearrangement of some of the gluten protein structure involves the exposure of positive charges (Italian Patent no.102015000084813, 2015) Positive molecules, namely cationic peptides, can exert a strong antibacterial effect because they supposedly act at the cytoplasmic membrane, leading to permeabilisation and eventually membrane disruption. Arginine and lysine residues play a major role in this process. Moreover, the interaction of antimicrobial peptides with anionic membrane phospholipids is a key factor in killing bacteria (Malanovic et al., 2015). To date, it is not clear whether the outer membrane of Gram-negative bacteria can exert a positive or negative effect. Our results suggest that the outer membrane did not play a role due to the significant antibacterial effect on both Salmonella Typhimurium and S. aureus.

Teichoic acid can act negatively on cationic peptides because they have a negative charge, catch positive molecules and decrease their potential towards cells (Malanovic et al., 2015). This idea could partially explain how GFB did not exert a negative effect on lactobacilli and bifidobacteria but did for S. aureus, and this difference might be the result of the unique structure of the cell wall and teichoic acids in lactobacilli, as described by Chapot-Chartier and Kulakauskas, 2014.

After this preliminary evaluation in strictly controlled conditions, we moved to a complex ecosystem to assess whether GFB can affect the evolution of heterogeneous microbiota. Two variables were assessed: the type of bread (CB and GFB) and the subject (coeliac or healthy people). The experiments were performed using 6 different subjects, and a negative control (batch culture

inoculated with faecal microbiota but not supplemented with any type of bread) was also added for each subject. In addition, we also assumed that the faecal microbiota could experience a qualitative-quantitative change per se (decrease or increase without supplementation due to a "donor effect") or after bread supplementation (change due a "bread effect"). Thus, we used a static approach to standardize the data to the negative control at each sampling time. For each group (healthy subjects, and coeliac subjects), the standardized values of the negative controls showed the donor effect not related to bread supplementation. Therefore, a negative value pinpointed that bread supplementation caused a decrease in the viable count, whereas a positive value highlighted an increase.

Some groups were chosen as tests to assess the effect of CB and GFB, such as lactobacilli, bifidobacteria, Bacteroidetes, eubacteria and clostridia. Bifidobacteria can produce vitamins (e.g., K, B12, biotin, folate, thiamine). The synthesis of secondary bile acids is mediated via Lactobacillus spp. and Bifidobacterium spp. (Nicholson et al., 2012). Moreover, Bifidobacterium spp. can also help prevent pathogenic infection through the production of acetate (Fukuda et al., 2011). To date, the role of Bacteroides is controversial: some authors have postulated a positive impact, whereas other researchers have found a strong correlation of these microorganisms with CD Nadal et al., 2007; Collado et al., 2007) and a possible role in the pro-inflammatory response [42], mucin degradation and increased permeability of the small intestine (El Asmar et al., 2002; Trompette et al., 2014). E. rectale (now reclassified as Agathobacter rectalis) (Rosero et al., 2015) is generally related to bifidobacteria, as it produces butyrate from acetate [46], with a beneficial effect on the host. The most valuable results were found for lactobacilli and bifidobacteria. In fact, the standardised values of lactobacilli from coeliac donors after 6 h were negative, suggesting that the lactobacilli population suffered a type of stress that was enhanced by bread supplementation. The shift from negative to positive values in coeliac subjects in the presence of GFB (sample F) suggests that the supplementation suddenly interrupted this stress and beneficially modulated the microbiota composition. However, this effect was reversible because the samples experienced a partial shift after 48 h, suggesting that a prolonged and a beneficial effect could also be the result of prolonged supplementation. The experimental data from the batch cultures also confirmed the ability of GFB to promote the growth of Lactobacillus spp. The shift was much stronger in coeliac donors probably due the unbalanced microbiota composition compared to healthy subjects.

Differently from the screening, GFB exerted a positive effect on the bifidobacteria numbers of coeliac donors, although the effect was found after only 48 h. This result, along with the death kinetics data in saline solution, suggests that bifidobacteria probably require prolonged supplementation.

The same approach was used to model and analyse SCFA profiles. There is growing recognition of the role of SCFAs in immune function and inflammation in tissues (Trompette et al., 2014).

Moreover, SCFAs can act as key sources of energy for colorectal tissues and bacteria and promote cellular mechanisms that maintain tissue integrity (Cummings and MacFarlane 1991; Topping and Clifton 2001; Donohoe et al., 2011). The data were quite variable, and bread supplementation did not exert a clear effect. To better understand this scenario, we decided to combine the SCFA data with the viable counts and pinpoint the changes at a global level.

Thus, the last statistical analysis (PCA) pinpointed a change in the ecosystem, and this effect was clearly distinguishable after 24 hours for coeliac donor + GFB sample, which moved from the region of coeliac people to healthy subjects. Thus suggests that GFB can cause a change in the whole ecosystem and exert a key role in the fight against the dysbiosis in coeliac people. This study provides new insights into the role of GFB on the qualitative-quantitative modulation of microbiota in simple or complex systems. The first step pinpointed that GFB has an important role in the prolongation of the survival of L. acidophilus and the antibacterial effect towards S. aureus and Salmonella Typhimurium. In a complex ecosystem, such as gut microbiota, GFB induced a beneficial modulation in terms of bifidogenic effects and on the growth of lactobacilli. Moreover, a final multivariate approach combining both the viable count and SCFA profile suggested that GFB causes a shift in the whole ecosystem. Therefore, this paper provides findings supporting the utilization of GFB to modulate the composition and metabolic profile of the intestinal microbiota in coeliac individuals.

The applicability of such changes were also evaluated in a 3 stage continuous in vitro colonic model. Our data have shown significant increases in bifidobacteria numbers in response to GFB administration in both healthy and coeliac subjects compared to the control bread. This effect could be related to the to the new detoxification method. Bifidobacteria are recognised as one of the most important bacterial groups associated with human health, providing beneficial effects in the large intestine (Gibson et al., 1994; Russel et al., 2011). The GFB fermentation also led to increases in *Bacteroides* spp. group and significant changes of *Clostridium* cluster XIVa group. Both groups may exert a detrimental effect on colon health due to an association with some metabolites. However, they also contain saccharolytic species which can produce large concentrations of beneficial SCFAs from sugars. SCFAs produced by gut microbiota in the colon have an important role. Butyrate is often associated as an energy source for the epithelial cells and acetate plays an important role in controlling inflammation and combatting pathogen invasion (Russel et al., 2013). In addition, SCFAs help to regulate sodium and water absorption, and can enhance absorption of calcium and other minerals as well as lower the colonic pH. GFB *in vitro* fermentation induced a modulation of the colonic microbiome with increased acetate and propionate levels, which was not observed with control bread in healthy donors. The best known metabolic pathway for acetate and propionate production from gut bacteria, involves the metabolism of polysaccharides. Acetate production occurs mainly through the

fructose-6- phospahte phosphoketolase pathway by bifidobacteria, and a greater production of this acid could be related to the increase of the bacteria (Miller et al., 1996). According to Hosseini et al. (2011), propionate can be produced from fermentable carbohydrates by two pathways. The first involves decarboxylation of succinate by the presence of *Bacteroides fragilis* and *Propionibacterium* spp. groups, while the second one is the acrylate pathway, in which pyruvate is first reduced to lactate by lactate dehydrogenase by the presence of some clusters of *Clostridial* groups. An increase in bifidobacteria, *Bacteroide*s and *E. rectale* groups was indeed observed during GFB fermentation.

GFB showed positive modulations of the microbiota composition, as well as increases in SCFA concentrations in both healthy and coeliac donors. Following GFB fermentation, beneficial modulations were seen in terms of bifidogenic effects in healthy and coeliac donors, as well as growth in *Clostridium* clusters XIVa+b numbers in coeliac subjects. This *in vitro* work provides encouraging findings supporting the utilization of GF products with a positive effect on the intestinal microbiota and metabolites composition in coeliac people.

Conclusions

This thesis aimed to investigate chemical, biochemical and technological characteristics of *Gluten Friendly*^{TM} grains of cereals and derived products.

This innovative method to detoxify gluten proteins (either the gliadins and the glutenins) from cereal grains that relies on the application of microwave energy for a few seconds to hydrated wheat kernels before milling: exposing the kernels to high temperatures for a short time lll induces a structural change in the gluten proteins (Lamacchia et al., 2015a,b) (Lamacchia et al., 2016; Landriscina et al., 2016). By this approach, the antigenic property of gluten is abolished (Lamacchia et al., 2016; Landriscina et al., 2016), and the *in vitro* immunogenicity of the most common epitopes of gluten is reduced (Lamacchia et al., 2015c).This modification does not compromise the nutritional and technological properties of the cereals, necessary to process semolina to pasta and flours to bread and other baked goods (Lamacchia et al., 2016). Furthermore the *in vitro* response of healthy and coeliac human faecal microbiota to gluten-friendly bread (GFB) was assessed (Bevilacqua et al., 2016), pinpointed that GFB prolonged the survival of L. acidophilus and exerted an antibacterial effect towards S.aureusand S. Typhimurium. Moreover, GFB modulated the intestinal microbiota in vitro, promoting changes in lactobacilli and bifidobacteria members in coeliac subjects. A final multivariate approach combining both viable counts and metabolites suggested that GFB could beneficially modulate the coeliac gut microbiome. These results were confirmed by an *in vitro* study performed by a three-stage continuous fermentative system on the intestinal microbiota and metabolites composition of healthy and coeliac individuals. Further investigations are actually

ongoing, at Roehampton University (London, UK), a prospective, parallel, placebo controlled, randomised, single centred study in 72 (30-50 years old) individuals to test the effects of “Gluten Friendly Bread” in coeliac disease patients.

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