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Dottorato di ricerca in Scienze degli Alimenti

Ciclo XXX

La qualità dei prodotti e della dieta senza glutine.

The quality of gluten-free products and diet.

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Abstract

Background and aim: Celiac disease (CD) is an autoimmune disease sustained by an inappropriate response to gluten in genetic predisposed individuals. The only treatment for CD is a lifelong strict gluten-free (GF) diet. A GF diet is a combination of naturally occurring GF foods and cereal-based foods formulated with GF substitutes of wheat, barley and rye.

The production of GF products with good technological quality and consumer acceptability is challenging for food manufacturers. Indeed, gluten determines the viscoelastic behavior of wheat-based doughs and other important rheological and sensory features of products. Over the years, various strategies have been proposed to overcome the lack of gluten. For instance, hydrocolloids are very effective in solving some of GF bread quality issues. However, besides technological and sensory aspects of GF products, the nutritional quality should deeply be explored.

Some population-based studies have pointed out several dietary imbalances among treated individuals with CD. Causes of such dietary imbalances in individuals with CD can be multiple. For instance, the focus on gluten exclusion and the several restrictions imposed by the GF diet.

In the previous years, different studies have been dealing with GF products and diet. However, there are some open questions that deserve attention.

In this view, the aims of this thesis were: i) to propose a simple tool to describe the nutritional profile of packaged GF bakery products in the perspective of leading their nutritional enrichment and comparing their nutritional quality to that of the gluten-containing (GC) counterparts; ii) to deepen the functional relationship between HPMC and hydration in GF bread making; iii) to address the role of Chia seeds and/or exuded mucilage as potential thickening agent and ingredient for nutritional enrichment in production of GF pasta, with similar characteristics to wheat pasta; iv) to study the dietary habits of a group of Italian individuals with CD with the aim of evaluating their adherence to a food pattern recognized for its protective role on the risk of major non-communicable disease.

Methods: i) The nutritional quality of the GF bakery products was evaluated with a score-based method, which combined information from the nutritional facts and the presence/absence of some nutritionally relevant ingredients in the product formulation. ii) The synergic role of HPMC and hydration in influencing the dough consistency and the textural features of GF bread was analyzed in a multilevel factorial model. Rheological parameters from GF dough and bread were collected. iii) Chia seeds and/or the exuded mucilage were added to a commercial GF pasta formulation. The cooking analysis of pasta was performed together with the characterization of the proximate composition, the *in-vitro* carbohydrate digestibility and the content of phenolic compounds. iv) A group of individuals with CD from the northern Italy was enrolled to measure the adherence of their dietary habits to a MD. The Italian Mediterranean Index was used to score their dietary habits.

Results: Regarding the GF bakery products and diet, our nutritional evaluation pointed out interesting findings. On one hand, the nutritional quality of GF bakery products was low according to the developed score. Interestingly, such a low quality was almost similar to that of the GC counterparts. In GF bread making, hydration was a major player in defining the consistency of a GF dough. However, the proper selection of HPMC, relying on its viscosity, may help to obtain good technological features of GF dough and bread. In GF pasta, both Chia seeds flour and exuded mucilage were useful to obtain a product with similar cooking and texture characteristics to those of wheat pasta. Furthermore, compared to a traditional commercial GF pasta, the inclusion of Chia seeds or mucilage raised the content of protein, dietary fibers and phenolic compounds.

On the other hand, the dietary habits of individuals with CD were far from MD pattern; they mainly had a high consumption of meat, processed meat and potatoes and a low intake of fruit and vegetables. Interestingly, such a low adherence to a MD was even lower than that of a group of healthy participants.

Conclusion: The quality of GF bread and pasta in terms of technological and nutritional characteristics can be ameliorated by the proper selection of HPMC and the inclusion of ingredients such as Chia seed or mucilage. However, GF products represent only a portion of food items in a GF diet. Accordingly, to improve the overall quality of a GF diet, dietary choices of individuals with CD should be better addressed towards a Mediterranean like pattern by healthcare professionals during the nutritional counseling.

Keywords: gluten-free products, gluten-free diet, dough, bread, hydroxypropylmethylcellulose, pasta, Chia seeds, nutritional quality, adherence to Mediterranean Diet.

Chapter 1

General introduction

1.1 Celiac disease

Celiac disease (CD) is a chronic systemic autoimmune disease sustained by an inappropriate response to gluten in genetic predisposed individuals (Zanoni et al., 2006). The term gluten is often used to encompass prolamins, specific storage ethanol-soluble proteins of wheat, rye and barley at the base of the characteristic immune response of CD.

Individuals with CD mainly develop an autoimmune injury to the mucosa of the small intestine resulting in a villous atrophy and a crypt hyperplasia. In addition, they present an increased number of immune cells in the lamina propria of the small intestine, which contribute to intensify the immune and inflammatory response. Therefore, classical gastrointestinal symptoms, such as diarrhea, steatorrhea and weight loss, due to malabsorption are prominent in individuals with CD (Guandalini & Assiri, 2014). Other symptoms, mainly extra-intestinal ones, such as non-autoimmune multiple organs injury, anemia, osteoporosis, and dermatitis herpetiformis, have been also reported (Zanoni et al., 2006).

Originally, it was thought that CD exclusively affected the European Caucasian population. However, an increase in individuals diagnosed for CD in other population groups from developing countries has been recently reported. Particularly, the overall prevalence of CD is nowadays about the 1-3% of the world's population (Fig. 1). Thus CD, previously considered a rare intestinal disorder, has become one of the most common genetic disorders resulting from the reciprocal interaction of environmental and genetic factors (Gujral, 2012).

To date CD is apparently growing at a faster pace than 20 years ago. However, such an increment may be primarily attributable to various factors, such as the spread of more sensitive diagnostic techniques and the progress of knowledge related to the understanding of the disease (Guandalini & Assiri, 2014). In addition, it has been observed a recent diffusion of the disease following the increased consumption of wheat-containing foods in countries that traditionally consumed naturally gluten-free (GF) cereals (Lionetti & Catassi, 2011).

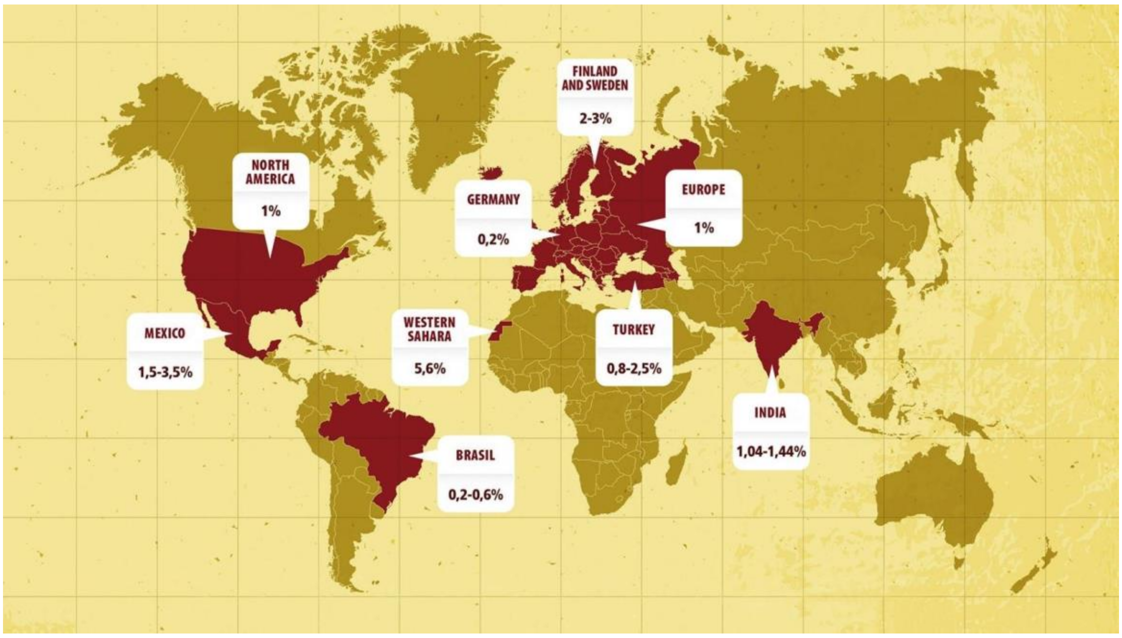

Fig. 1 – World prevalence of CD (*Courtesy of the Dr. Schär Institute*).

Genetic bases and environmental triggers

Genetics play a strong role in development of CD, as confirmed by the disease concordance in monozygotic twin (Gujral, 2012) and its occurrence in about 10% of first degree relatives (Volta, Caio, Stanghellini, & De Giorgio, 2014).

Individuals with CD present specific alleles of the human leucocyte antigen (HLA), a gene system encoding for the proteins of the major histocompatibility complex II located on the immune cell surface. In particular, CD has been strongly associated with HLA-DQ2 or HLA-DQ8 haplotypes (Nanayakkara et al., 2013). In fact, HLA genes are responsible for up to the 40% of the genetic risk (P. H. R. Green & Cellier, 2007) of the disease.

The rest of the genetic risk is attributable to non-HLA CD risk loci. These loci, by harboring genes associated with the control of innate immunity and adaptive immune function can influence particularly T-cell activation and development (Verdu, Galipeau, & Jabri, 2015).

Interestingly, genome-wide association studies identified common shared genetic basis between CD and other autoimmune disease. For instance, HLA-DQ8 allele is considered a strong susceptibility factor for the development of type-1 diabetes in individuals genetically predisposed to CD. HLA-DQ2 allele is the genetic basis for the increased prevalence of CD in patients with type-1 diabetes (Akirov & Pinhas-hamiel 2015). In addition, several shared HLA and non-HLA loci, containing various alleles that may confer risk both for CD and rheumatoid arthritis, have been recently identified (Zhernakova et al., 2011).

The exact etiology of CD is still to be clarified. Nowadays, researchers agree about the primary role of genetic predisposition and the loss of tolerance by the immune system to gluten peptides as major triggering factors of the disease. However, it is worth to underline that about the 30% - 40% of the general population carries the HLA-DQ2 or HLA-DQ8 alleles, but less than the 3% of these people develop a celiac condition (P. H. R. R. Green & Jabri, 2006).

Therefore, along with the genetic predisposition, various environmental causes have been proposed as co-factors in the etiology and progression of CD. For instance, it has been hypothesized that intensive drug treatment (i.e. prolonged antibiotic therapies) and serious intestinal viral infections may impair or negatively influence the immune system tolerance to food antigens. These factors could contribute to the loss of gluten tolerance in genetically predisposed individuals (Leonard, Camhi, Huedo-Medina, & Fasano, 2015).

Furthermore, infant-feeding practices, such as the breastfeeding duration or the timing of gluten introduction, have been long considered as major contributors of the CD onset in genetically predisposed children. However, the observations on the optimal breastfeeding duration or the correct introduction of gluten in children at risk of CD have recently been reviewed and no evidence supports a causal relationship between infant feeding practice and onset of CD (Silano, Agostoni, Sanz, & Guandalini, 2016).

In the last few years, researchers focused on the possible role of the small intestinal microbiota and the altered expression of some specific and non-specific CD-risk genes in the etiopathology of CD.

In particular, it has been proposed that the irregular immune response produced by an altered expression of these specific and non-specific CD-risk genes may disrupt the host-gut microbiota immune tolerance and, in turn, trigger an inflammatory cascade in individuals predisposed to CD. Although the exact progression remains unclear, such inflammatory cascade is believed to contribute to the loss of gluten tolerance in the small intestine (María Carmen Cenit, Olivares, Codoñer-Franch, & Sanz, 2015).

The role of gluten in the etiology and pathogenesis of CD

Gluten prolamins contained in foods are rich in proline and glutamine residues that confer resistance to the proteolytic action of small intestine enzymes. The incomplete digestion of gluten prolamins leads to the appearance of several gluten derived peptides (Fig. 2) that can exert various actions in the intestinal mucosa by interacting with the cells of the adaptive immune system (Gujral, 2012).

It has been proposed that some gluten derived peptides can induce enterocytes of individuals with CD to release a protein called zonulin. This protein is able to increase the permeability of the small intestinal barrier (Fasano, 2011). Interestingly, other environmental triggers of zonulin release are the intestinal infections and the inflammatory cascade resulting from the loss of immune tolerance to antigens of gut bacteria (Lammers et al., 2011). In addition, the gluten derived peptides can also stimulate the release of several local chemokines, particularly the interleukin-15 (IL-15). These chemokines arouse intra-epithelial lymphocytes against enterocytes contributing to the onset of the lining cells damage (Benahmed et al., 2007).

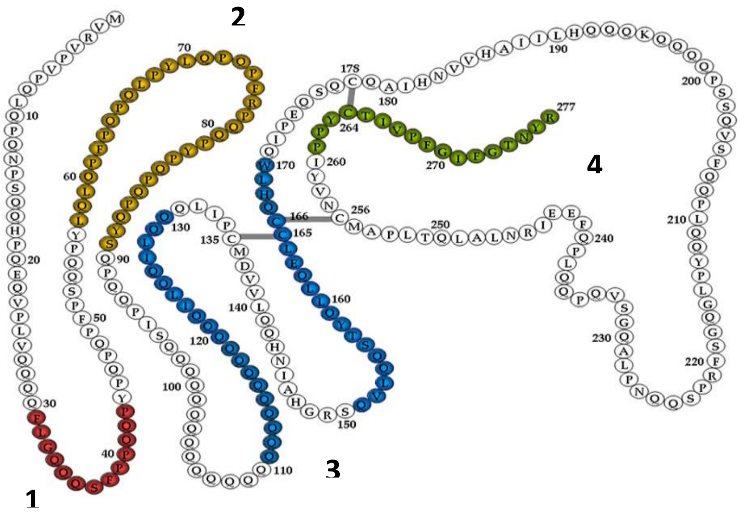

Fig. 2 – Gluten derived peptides involved in the etiology and pathogenesis of CD.

1. Cytotoxic activity (Maiuri et al., 1996); 2. Immunomodulatory activity (Shan et al., 2002); 3. Zonulin and gut permeating activity (Lammers et al., 2008); 4. Enterocyte's zonulin receptor CXCR3 ligand and chemokine release factor (Lammers et al., 2011).

Following the increased permeability of the small intestinal barrier, gluten derived peptides can cross and accumulate under the lining cells. Then, these peptides can interact with the tissue transglutaminase 2 (tTG2) released by the damaged enterocytes. The tTG2 can deaminate the gluten derived peptides, mainly the 33-mer peptide, which contains a characteristic aminoacidic sequence recognized by the antigen presenting cells (APCs) expressed on the surface HLA-DQ2 or HLA-DQ8 proteins (Lionetti & Catassi, 2015). APCs display the modified 33-mer peptides to the lymphocytes T-helper, which can spur natural killer T-cells to attack enterocytes. At the same time, B lymphocytes start to differentiate and produce i) IgG antibodies against deaminated gluten peptides (DGP-AGA); ii) IgA autoantibodies against the endomysium (EMA) and iii) IgA autoantibodies against tissue transglutaminase (tTGA) (Fasano, 2009) (Fig. 3).

The main consequence of this sequence is a persistent inflammatory condition of the small intestine mucosa, which causes the disruption of lining cells and the degeneration of the tissue architecture. Such degeneration leads to villous atrophy, crypt hyperplasia, infiltration of lymphocytes in the epithelium and, in turn, a reduced absorption of nutrients from diet (Guandalini & Assiri, 2014).

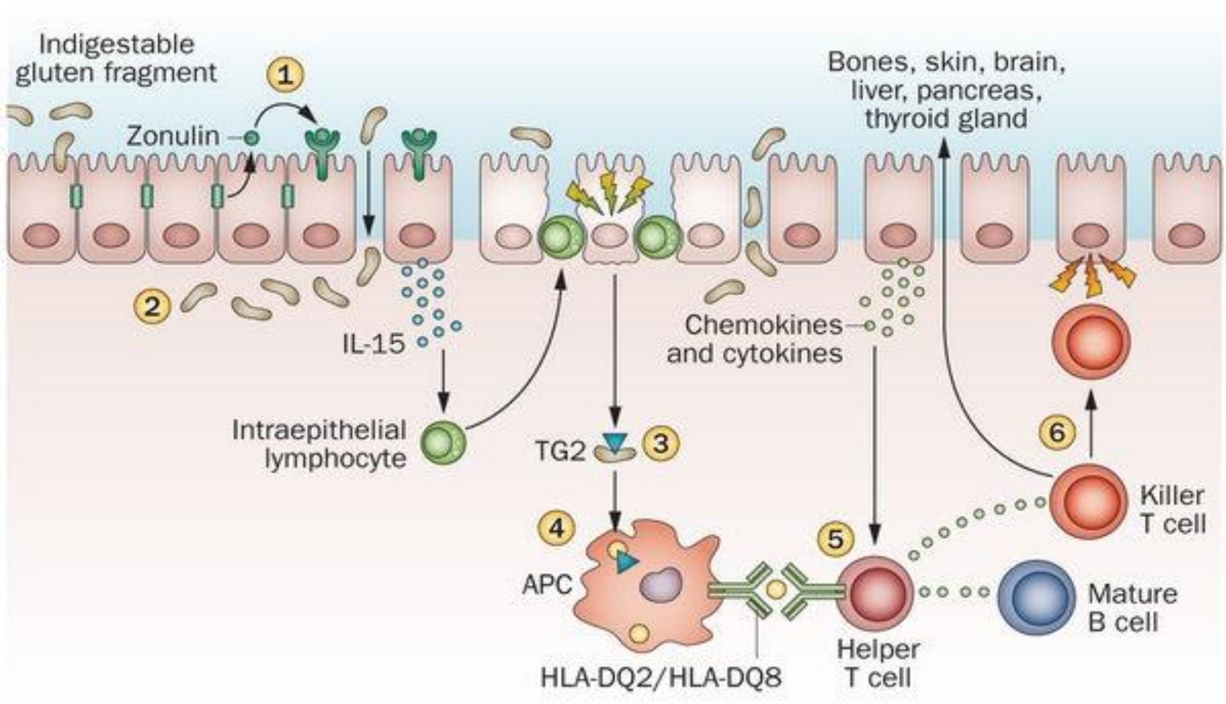

Fig. 3 – Etiology of CD. Mechanism of the intestinal and extra-intestinal manifestations (Leffler, Green, & Fasano, 2015).

Diagnosis criteria of CD

The diagnosis of CD begins with the typical and atypical intestinal events reported by patients, including abdominal pain, intestinal bloating, substantial loss of weight and diarrhea or constipation. Moreover, some extra-intestinal symptoms, such as anemia and osteoporosis, are nowadays part of the first diagnosis process. The tests for the presence of typical serological (DGP-AGA, EMA and tTGA) and histological (villous atrophy and crypt hyperplasia) features completes the assessment of the clinical picture of the disease (Volta & Villanacci, 2011). Interestingly, CD has been reported more common among women compared to men. However, this difference is mainly attributable to the early diagnosis of CD in women due to the manifest consequences of the disease, such as anemia and reduced bone density (P. H. R. Green, 2009).

The European Society for Pediatric Gastroenterology, Hepatology, and Nutrition published in 2012 an update on the diagnostic criteria for CD. Briefly, diagnosis is mainly based on the measurement of tTGA and EMA content in blood. The DGP-AGA blood measurement is usually performed in patients that are negative for the tTGA and EMA, but with a strong suspicion of CD due to their clinical symptoms, or in case of a patient younger than 2 years old (Husby et al., 2012).

It is worth to underline that these serological tests have been extensively validated and are used both in the CD diagnosis and the follow-up of individuals with CD (Guandalini & Assiri, 2014).

Genetic tests for the identification of the HLA-DQ2/DQ8 is a useful tool to rule out CD, *i.e.* confirming the value of positive serological testing or in case of negative or uncertain results.
In children, a genetic test can confirm the strong suspicion of CD, avoiding blood antibodies measurements and small intestine biopsy (Husby et al., 2012). A biopsy of the small intestinal mucosa may be recommended in adult patients to confirm the clinical relevance of a positive serological test for CD.
In recent years, a progressive decline in the use of the intestinal biopsy as a diagnosis tool for CD is evident, particularly in presence of high antibody levels and clear clinical manifestations (Husby et al., 2012; Volta & Villanacci, 2011).
After diagnosis of CD, the only treatment to prevent and contrast clinical manifestations and allow the mucosal healing of the small intestine in individuals CD is a lifelong strict exclusion of gluten from the diet (Newnham, Shepherd, Strauss, Hosking, & Gibson, 2016).

1.2 The gluten-free diet

Individuals with CD can exclude gluten from their diet by combining naturally occurring GF foods, including vegetables, fruits, meat, fish, dairy, legumes and nuts, and cereal-based foods formulated with GF grains that are substitutes of wheat, barley and rye. There are many naturally GF cereals, such as rice, corn, buckwheat, millet, quinoa, permit to individuals with CD that can be used to produce GF products.
Since 1979 the Codex Alimentarius proposed a standard to describe the products intended for individuals with CD. With such a document, the definition of GF products and foods with a reduced content of gluten was established. Over the years, several legislations have inspired their food labelling systems to this standard. In European Union (UE), the Regulation No. 1169/2011 and the Commission Implementing Regulation No. 828/2014 define the specific requirements for the provision of information to consumers on the absence or reduced presence of gluten in food (Simón et al., 2017). In particular, it has been stated that exclusively foods containing less than 20 parts per million (ppm) of gluten can be claimed as GF foods and foods with a gluten content from 20 ppm to 100 ppm can only be named very low in gluten.
The inclusion of oats among safe grains in the diet of individuals with CD has been long a debated issue (Mäki, 2014). Oats and oatmeal are traditional ingredients for the preparation of foods in many countries, especially in northern Europe (Størsrud, Hulthén, & Lenner, 2003). In addition, oats is nowadays considered a *superfood* due to its high content in vitamins and minerals and the presence of bran beta-glucans involved in controlling the serum lipids level (Biörklund, van Rees, Mensink, Onning, & Önning, 2005; Butt, Tahir-Nadeem, Khan, Shabir, & Butt, 2008).

Oats could be a suitable ingredient for the production of nutrient GF cereal-based products also in view of its low content of prolamins (Rosell, Barro, Sousa, & Mena, 2014). However, in many European countries and in North America oats is still harvested, stored and milled in facilities that manufacture also wheat and barley, thus resulting in cross-contamination of commercial oats with gluten (Pietzak, 2012).

Clinical studies suggest that oats' prolamins have a minimal stimulating effect on the immune system of the majority of individuals with CD. However, a small proportion of individuals with CD experiences adverse immune effects after oats ingestion (P. H. R. Green & Cellier, 2007). Consequently, oat is not yet considered a safe grain and many dietitians allow its gradual introduction only into the diet of well-controlled individuals with CD.

Adherence to a GF diet

A long-term adherence to a GF diet can ensure a good recovery from the intestinal manifestations of CD in individuals with CD. However, following a GF diet may entail various difficulties and sometimes individuals fail to exclude gluten from the diet. In fact, although they are aware on the risk of gluten ingestion, it has been estimated that the 30-40% of individuals with CD do not have a strict adherence to GF diet (Biagi et al., 2009).

Several studies have investigated the causes of such a lack of adherence and the impact that the GF diet has on the quality of life of children (Case, 2005), adolescents (Kautto et al., 2014) and adults (Zarkadas et al., 2013) with CD. Indeed, many factors, including personal and cultural influences and social interaction, can influence the ability of individuals with CD to modify their food habits (Zarkadas et al., 2013). For instance, difficulties due to a certain degree of embarrassment were highlighted in children and adolescents who have to bring GF foods to school, parties or friends house (Case, 2005). In adults with CD, instead, the change of long established dietary habits seems to play a major role in the low adherence to a GF diet (Zarkadas et al., 2013).

In many countries, individuals with CD can rely on support groups and societies, which promote conscious food choices, especially in the early stage since the GF diet establishment. In this sense, it has been pointed out that young people with CD may benefit from an extra guidance to exclude gluten and an additional support, even outside of family, to discover GF alternatives (Kautto et al., 2014).

Actually, it is a matter of fact that most of the daily consumed cereal staple foods, such as bread, pasta and breakfast products, are traditionally produced with gluten-containing (GC) cereals. In addition, gluten is often used as a food additive in many food preparations not related with cereals.

Further difficulties in following and adhering to a GF diet have been related to the price of GF foods (Singh & Whelan, 2011) and the availability of acceptable substitutes for the GC cereal-based foods, especially travelling and eating out of home. Furthermore, despite nowadays the variety and the availability of these products have been improved compared to few years ago (Macculloch, 2014), for many individuals with CD, even after years, following a GF diet still remains a substantial burden (Zarkadas et al., 2013).

Nutritional quality of GF diet

The nutritional status of non-treated and newly diagnosed individuals with CD is dependent on the degree of small intestinal mucosa injury, which affects the absorption capacity of macro and micronutrients. In fact, before diagnosis and treatment with a GF diet, the malabsorption of zinc, iron, folate and calcium is common due to the primary injury located in the proximal small intestine. Together with the progression of the inflammation, the cascade of the injury occurs along the distal small intestinal mucosa and the malabsorption of nutrients affects the carbohydrates, lipids and fats soluble (*i.e.* A and D-vitamins) and water soluble vitamins (*i.e.* B-vitamins) (Niewinski, 2008; Wierdsma, van Bokhorst-de van der Schueren, Berkenpas, Mulder, & van Bodegraven, 2013).

After the institution of a GF diet, several factors, including the duration of untreated disease, the extent of damage to the small intestinal mucosa and then the degree of malabsorption of certain nutrients, may continue to influence the nutritional status of individuals with CD (Theethira & Dennis, 2015).

For the majority of individuals with CD compliant with a GF diet, the resolution of the inflammatory condition and the recovery of the villous atrophy are reported. Although clinical observations report that time to mucosal healing may occur in more than six months (Collin, Maki, Kaukinen, & Collin P., 2004; Rubio-Tapia et al., 2010) a betterment of the absorption of nutrients, in particular vitamins and minerals including zinc, calcium, iron and vitamin D, is usually observed (Hallert et al., 2002).

The recuperation of the absorption capacity, dependent on mucosal healing rate, further permits a general improvement of the anthropometric parameters, such as body weight, body mass index (BMI), fat and bone mass, in treated individuals with CD when compared to newly diagnosed or non-treated individuals (Theethira, Dennis, & Leffler, 2014).

However, the general assumption that the simple institution of a GF diet would be sufficient to ensure a safe and nutritionally adequate dietary regimen to individuals with CD is changing in the last few years. In fact, it is worth to consider that celiac population is not exempt from the shift of general population towards less healthy dietary habits, evidenced by an increase in the number of overweight and obese individuals with CD (Kabbani et al., 2012; Theethira et al., 2014; Ukkola et al., 2012).

In the last two decades, some population-based studies pointed out various dietary imbalances among individuals with CD treated with a GF diet. The major studies are summarized in Table 1. In these studies, the dietary habits of individuals with CD were usually compared to a group of non-celiac controls in order to understand how those were different to those of the general population. Moreover, the nutritional quality of the GF diet of those individuals was usually evaluated based on the local national dietary recommendations.

Table 1 – Information about macronutrients and micronutrients intake in individuals with CD treated with a GF diet. Dietary habits of individuals with CD were compared with those of non-celiac individuals. The nutritional quality of the GF diet of those individuals was also evaluated using the local dietary recommendations.

Authors	Study population, dietary tools, control group	Significant results on dietary habits
Mariani et al. (1998)	47 **adolescents** with CD from the central Italy: 10 men and 37 women, mean age, 14.9 years. Dietary information of celiac individuals was: - Recorded by a 3-days food diary. - Compared to that of a control group composed of 47 non-celiac adolescents from the same area. - Compared to Italian recommended daily intakes of energy and nutrients (Livelli di Assunzione Raccomandata di energia e nutrienti, LARN – 1996) and United States recommended daily allowances (RDAs).	*Energy*: In individuals with CD, both men and women, the total intake was under the Italian and American recommended levels. However, compared to the energy intake of non-celiac controls there were no differences. *Fat*: the intake level for individuals with CD was similar to that of non-celiac controls. However, both the groups of subjects introduce more energy from fat than recommended by LARN and RDAs. *Carbohydrate*: individuals with CD introduced less energy from carbohydrates than controls. Moreover, the intake of carbohydrates for celiac individuals and controls was below the LARN and RDAs percentage. The intake of *dietary fiber* for celiac individuals and non-celiac controls was similar and lower than that recommended by LARN and RDAs. *Proteins*: the relative contribution of proteins to the total energy intake was lower for celiac individuals than controls. However, both the groups of subjects consume more proteins than those recommended by LARN and RDAs. *Micronutrients*: intake of iron and calcium for celiac individuals and non-celiac subjects was similar and lower than that recommended by LARN and RDAs.
Grehn et al. (2001)	49 **adults** with CD from Sweden: 18 men and 31 women, aged 45-64 years. Dietary information of celiac individuals was:	*Energy*: in individuals with CD, both men and women, the total intake was within the NNR and similar to that of controls. *Fat*: the intake level for celiac individuals was similar to that of the controls, but higher than the NNR recommendations.

	- Recorded by a 4-days food diary for celiac individuals and 7-days food diary for the control group. - Compared to that of a control group composed of 498 non-celiac adults from the Swedish national dietary survey 1989. - Compared to the Nordic Nutrition Recommendations (NNR, 1989).	*Carbohydrate*: women with CD introduced more energy from carbohydrates than controls, whereas in the case of men there were not significant differences. However, the intake of carbohydrates for celiac individuals and controls was within the NNR percentage. Celiac individuals and controls consume less *dietary fiber* than that recommended, but the intake for celiac individuals was lower than controls. *Proteins*: the relative contribution of proteins to the total energy intake was similar for celiac individuals and controls and was within the recommended percentage. *Micronutrients*: intake of folate, vitamin E and selenium was lower than recommended for celiac individuals and controls.
Hopman et al. (2006)	132 **young** celiac individuals from Netherlands: mean age, 16 years. Dietary information of celiac individuals was: - Recorded by a 3-day food questionnaire. - Compared to food habits of the non-celiac Nederland's population (General Dutch Population dietary intakes). - Compared to the Dutch dietary recommendations (DRDA) and the American dietary recommendations (RDAs).	*Energy:* intake for celiac individuals was higher than the DRDA but similar to that of the general population and the RDAs. *Fat:* only saturated fat intake was higher than that of DRDA and general population. *Carbohydrate*: intake for celiac individuals was higher than general population but similar to DRDA and within the range of the RDAs. *Dietary fiber* intake was lower than the reference intakes of DRDA, general population and RDAs. *Proteins*: intake for celiac individuals was lower than the general population but within the range of the DRDA and RDAs. *Micronutrients*: for celiac individuals B-vitamins intakes reached or exceed the general population as well as the DRDA and the RDAs levels. Calcium and iron intake was similar to general population and DRDA but lower than RDAs.
Kinsey et al. (2008)	47 **adults** with CD from UK, 12 males and 35 females, mean age, 58.6 years. Dietary information of celiac individuals was: - Recorded by a 3-day food diary.	*Energy:* in individuals with CD the total intake was less than that recommended and similar to that of non-celiac controls. *Fat:* celiac individuals did not exceed the recommended percentage and their intake was lower than controls.

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<tr><td></td><td>- Compared to that of a control group representative of the UK general population (National Diet and Nutrition Survey – NDNS).
- Compared to dietary reference value for UK population (DRV - 1991).</td><td colspan="2">Carbohydrate: for celiac individuals the intake was not significantly different from that recommended and similar to that of non-celiac controls. Dietary fiber: for celiac individuals the intake was similar to controls and lower than recommended.
Proteins: celiac individuals exceeded the dietary recommendations and the intake was higher than controls.
Micronutrients: vitamin D and calcium intake was lower than that recommended.</td></tr>
<tr><td>Öhlund et al. (2010)</td><td>25 children with CD from Sweden, aged 4-17 years.
Dietary information of celiac individuals was:
- Recorded by a 5-day food diary.
- Compared to healthy children from Riksmaten – children 2003 (Swedish national food survey 2003 on a group of more than thousand 4 years old children).
- Compared to Nordic Nutrition Recommendations (NNR – 2004).</td><td colspan="2">Energy: for celiac individuals the amount was less than that recommended.
Fat: celiac individuals met the recommended percentage of total fat. Saturated fat intake of celiac individuals was higher than that recommended whereas polyunsaturated fat intake was lower than that recommended. The intake of saturated and polyunsaturated fat resembles that of the control group.
Carbohydrate: celiac individuals met the recommended percentage. Sucrose intake of celiac individuals was higher than that recommended, whereas dietary fiber intake was lower than that recommended. Healthy children consumed more dietary fiber than celiac children.
Proteins: celiac individuals met the recommended percentage. However, their consumption was lower than that of healthy children.
Micronutrients: a poor vitamin D status, common in the northern countries, was found both in healthy and celiac children. Magnesium deficiency has been reported for celiac children.</td></tr>
<tr><td>Wild et al. (2010)</td><td>Adult: 31 males (aged 19-64 years) with CD and 62 females (aged 35-69 years) with CD.
Dietary information of celiac individuals was:</td><td>Males
Energy: intake in celiac males was higher than NDNS males group.</td><td>Females
Energy: intake in celiac females was higher than that of NDNS females group but not with respect to that of UKWCS group.</td></tr>
</table>

	- Recorded by a 5-day food diary based on EPIC food survey for the portion sizes. - Compared to 195 males and 256 females from the NDNS – 2001. - 708 non-celiac females from the UK Women's Cohort Study (UKWCS) were further used as the reference population. - Compared to Recommended nutrients intake (RNI – UK).	*Fat:* intake in celiac males was higher than NDNS males group. *Carbohydrate:* intake in celiac males was higher than in NDNS males group. *Dietary fiber* intake was similar to that of the NDNS group. Celiac males consume more fiber than the RNI. *Extrinsic sugar* intake was higher than in NDNS. Few individuals met the recommended intake of RNI. *Proteins:* intake in celiac males was similar to that of NDNS males group. *Micronutrients:* intake in celiac males was similar to that of NDNS males group.	*Fat:* intake in celiac females was higher than that of NDNS females group but not with respect to that of UKWCS group. *Carbohydrate:* intake in celiac females was higher than in NDNS females group but not with respect to that of UKWCS group. *Dietary fiber* intake was lower than that of UKWCS group. Celiac females consume more fiber than the RNI. *Extrinsic sugar* intake was higher than in NDNS and UKWCS. Few individuals met the recommended intake of RNI. *Proteins:* intake in celiac females was higher than in NDNS females group but not with respect to UKWCS group. *Micronutrients:* magnesium, iron, zinc, manganese, selenium and folate in celiac females were lower than in UKWCS group.
Zuccotti et al. (2013)	18 Italian **children** with CD from northern Italy, mean age 4.2 years. Dietary information of celiac individuals was: - Recorded by a Food Frequency Questionnaire (FFQ).	*Energy:* intake in celiac children was higher than that of non-celiac children. *Fat:* intake in celiac children was lower than that of non-celiac children. Intakes in both groups exceeded the LARN recommendations. Intakes of *saturated, monounsaturated* and *polyunsaturated* fats did not differ between the two groups. *Carbohydrate:* intake in celiac children was higher than that of non-celiac children and in contrast to them met the LARN recommendations. *Sugars* intake was not different	

	- Compared to that of 18 non-celiac children from the same geographical area. - Compared to Italian recommended daily intakes of energy and nutrients (Livelli di Assunzione Raccomandata di energia e nutrienti, LARN – 1996).	between the two groups but it was higher than that of LARN recommendations. *Dietary fiber* intake was similar between the two groups. *Proteins:* both celiac children and non-celiac children had a greater consumption than the upper LARN recommendation. However, celiac children consumed higher amount of proteins than non-celiac children. *Micronutrients:* intake of vitamin D in celiac children was lower than that of non-celiac children. Intakes of calcium, B-vitamins, iron and magnesium did not differ. Compared to LARN, the overall micronutrients intake did not meet the LARN recommendations.
Churruca et al. (2015)	54 celiac **adult** women from Spain, mean age 34 ± 13 years. Dietary information of celiac individuals was: - Recorded by a 24 h food recall and a FFQ. - Compared to the Dietary reference intakes (DRI – Spain 2010) and to the Spanish Society of Community Nutrition (SENC). - Compared to Spanish reference adult women (1734) population.	*Energy:* intake in celiac women was below the DRI value and lower than the control group. *Fat:* intake exceeded the DRI for both the celiac and the control women. Intake in celiac women was lower than that of the control group. *Carbohydrate:* intake in celiac women was lower than that of the control group. Both groups were within the value of the DRI. *Dietary fiber* intake was lower in celiac women than in the control group. *Protein:* intake in celiac women was similar to that of the control population and higher than the DRI. *Micronutrients:* the intake of vitamin D, E and folate, iron and calcium in celiac women was under the DRI value as the control women group. Intakes of vitamin E, niacin as well as magnesium and selenium were lower in celiac women than in the control group.

In the light of the studies reported in Table 1, it is possible to hypothesize several causes behind the observed nutritional imbalances of individuals with CD. Shepherd & Gibson (2013) have recently suggested that during the early years since the establishment of the GF diet, the focus on the exclusion of gluten may lead individuals with CD to neglect the nutritional quality of their food choices. However, although some differences among the studies exist, remarkable nutritional imbalances have been identified also in individuals with CD following a long-term GF diet (Kautto et al., 2014; Vici, Belli, Biondi, & Polzonetti, 2016). Therefore, researchers hypothesized that some behavioral factors, non-dependent to the disease, as well as an inadequate nutritional counselling may influence the nutritional status of individuals with CD (Bardella et al., 2000; Kabbani et al., 2012; Ukkola et al., 2012). In particular, it has been stressed that the GF diet, being a restrictive regimen, might contribute to the nutritional imbalances observed in individuals with CD (Miranda, Lasa, Bustamante, Churruca, & Simon, 2014; Wild et al., 2010).

When the gluten exclusion is pursued, staple cereal-based foods, such as baked products and pasta, have to be produced with GF ingredients, of which the most common are starches from potatoes and cassava and refined flours from rice and corn (Matos & Rosell, 2014). It was pointed out that starches and refined flours can negatively influence the content of nutrients, such as dietary fibers and minerals (i.e., iron and vitamins), of cereal-based GF products (Foschia, Horstmann, Arendt, & Zannini, 2016; Thompson, 2000; Yazynina, Johansson, Jägerstad, & Jastrebova, 2008). In addition, it has been recently observed that, due to the bland flavor and the scarce palatability of most of the commercial available GF products, food manufacturers add fat, especially saturated fat and salt, to the GF formulations (Miranda et al., 2014).

Moreover, the presence of high digestible starches and carbohydrates in the formulation of GF products may influence the postprandial glycemic response in individuals with CD. Such aspect is of particular interest since the high prevalence of CD in patients with a diagnosed type-1 diabetes (Scaramuzza, Mantegazza, Bosetti, & Zuccotti, 2013).

As aforementioned, the principal food group affected by the gluten exclusion is that of cereals and cereal-based foodstuffs (Thompson, 2009). Such a food group covers up to the 38% of the daily energy intake in the Italian population, for instance (Sette et al., 2013). Cereal-based foods produced with GC flours from wheat, barley or rye are considered important sources of minerals and vitamins, especially vitamin B. Moreover, often these products contain wholegrain flours, which ensure an adequate contribution to the daily intake of dietary fibers (Sette et al., 2013).

Therefore, the nutritional quality of GF cereal-based products is pivotal for individuals with CD. In fact, these individuals habitually introduce up to the 36.6% of their daily energy intake from commercially available GF cereal-based foods (Zuccotti et al., 2013). In this frame, an improvement of the nutritional quality of the ingredients, of which the GF products are composed, is necessary to contribute to the correct nutritional balance of the GF diet. However, to reach this goal, a holistic approach that join together technological techniques and nutritional needs is necessary.

1.3 The gluten-free products

Over the centuries, cereal-based products obtained from rice, corn, sorghum, wheat, barley, oat and rye have been staple foods spread all over the world (Salamini, Ozkan, Brandolini, Schäfer-Pregl, & Martin, 2002). In particular, among all the foods produced from cereals, baked products, and in particular bread, have long been leading in human nutrition (Foschia et al., 2016).

From a technological point of view, wheat is an ideal cereal for the production of baked goods and other cereal foods such as pasta. In fact, in a wheat-based system, when mixing the ingredients, the addition of water allows the protein of the gluten complex to form a network that wraps the hydrated starch granules and other flour components. Such gluten network determines the viscoelastic behavior of the wheat-based doughs as well as other important rheological features, including strength and extensibility resistance, mixing tolerance and gas holding ability during proofing (Lazaridou, Duta, Papageorgiou, Belc, & Biliaderis, 2007). The gluten network ensures a good volume expansion during proofing and a softer crumb in bread and in other leavened products and produces a cohesive structure in pasta (Gómez & Sciarini, 2015).

With this in mind, it is clear that going to exclude gluten from doughs, the food manufacturers are faced with a major technological challenge in producing cereal-based GF products.

Technologically, a GF system differs from a wheat-based system because of parameters, such as the consistency and viscoelastic properties of dough. In fact, a GF system contain proteins with poor structural properties unable to form a gluten-like network. Therefore, in absence of structuring agents the viscoelastic properties of a GF system rely only on the weak intermolecular interactions of a continuous phase established by the hydrated starch granules. As a result of such weak binding forces, the GF system has low cohesiveness and stability, low viscosity, poor mixing tolerance and low handle ability (Crockett, Ie, & Vodovotz, 2011; Foschia et al., 2016; Sivaramakrishnan, Senge, & Chattopadhyay, 2004). These characteristics are responsible of rheological problems of GF doughs and of several post-baking consequences, such as dense bread with a low-volume, high crumbly texture and poor mouthfeel, which negatively affect the product acceptability by the consumers (Crockett et al., 2011).

The formulation of cereal-based GF products

The formulation of cereal-based GF products, such as bread and pasta, is rapidly changed following the technological innovations that characterized the last years.

Along with gluten, starch significantly contributes to texture, appearance and overall acceptability of cereal-based products (Horstmann, Belz, Heitmann, Zannini, & Arendt, 2016). In GC cereal products such a remarkable contribution is normally fulfilled by the high amount of starch present in wheat flour. For this reason, wheat starch was initially the major ingredient in the preparation of GF cereal products. However, due to the problem of cross-contamination with gluten, wheat starch employment was reduced and other starch sources, such as rice, corn, cassava and potato were considered (Gómez & Sciarini, 2015). Nowadays, starch isolation methods from wheat have been improved, thus ensuring a final product containing less than 20 ppm of gluten.

Afterwards, GF flours from the major GF cereals have been introduced. In particular, rice flour, owing to its easy availability, hypoallergenic properties, high digestibility, bland taste and white color, is nowadays one of the most important raw materials in cereal-based GF products manufacture (Phongthai, D'Amico, Schoenlechner, & Rawdkuen, 2016). Although less frequently used than rice, white corn, millet and recently sorghum have been also included in the formulation of cereal-based GF products. The peculiarity of these GF flours is that their starch proved to be a good substitute of the appreciated technological features of wheat starch for the production of cereal-based GF products and pasta (Gómez & Sciarini, 2015).

Beside the basic ingredients (flour, starch, water, salt, sugar and yeast), emulsifiers, such as glycerol mono-stearate, was firstly proposed as food additives in order to mimic the gluten function (Jongh, 1961). Subsequently, in the last two decades, the focus of the research in GF cereal science moves towards the study of other food additives, for example proteins from animal (eggs and milk) and vegetable (soy) sources (Lazaridou et al., 2007; Renzetti & Rosell, 2016). However, due to the potential allergenic risk toward proteins from eggs (white eggs), milk (whey) and soy, the hydrocolloids have drawn the interest of the researchers working on GF products (Anton & Artfield, 2008).

Hydrocolloids

Hydrocolloids are a family of compounds that chemically belong to polysaccharides. They can be classified according to their origin. Polysaccharide hydrocolloids can be extracted from several natural sources: plants, such as marine algae, tree leaves or cortex; plant foods including fruit, root vegetables, cereal bran and seeds mucilage; bacteria biofilms.

Plant foods hydrocolloids are known as agar-agar, carrageen, pectin, oats' beta-glucan, gum arabic, psyllium and xanthan gum. Hydrocolloids can also be obtained by chemical and biochemical synthesis and cellulose derivatives, such as hydroxypropylmethylcellulose (HPMC) and carboxymethylcellulose (CMC), are a good example (Houben, Höchstötter, & Becker, 2012).

Polysaccharide hydrocolloids have long been used as food additives in traditional wheat-based baked goods, especially in bread making and sponge cake formulations, in order to obtain additional water binding and thus producing moister and softer products with prolonged shelf-life (Crockett et al., 2011).

In GF bakery preparations, hydrocolloids have been proposed as effective agents in order to mimic the effect of gluten on the viscoelastic and cohesive behavior of doughs. Particularly, hydrocolloids can build a three dimensional structure with defined water binding properties similar to that of the gluten network (Crockett et al., 2011). This structure is able to wrap the swelled starch granules and the gas bubbles, positively influencing the stability of the GF system.

In GF bread making, hydrocolloids can increase the gas retention capacity by rising the viscosity of doughs and producing a bread with good loaf volume, a parameter known to have a relevant impact on the product acceptance by consumers (Crockett et al., 2011; Houben et al., 2012). It is worth to underline the ability of hydrocolloids in controlling the moisture content and working as surfactants. Such an important ability in cereal-based GF products as bread may delay the staling rate and positively influence other quality parameters, as firmness and loaf volume (Crockett et al., 2011).

Recently, hydrocolloids as the HPMC, as a class of compounds, have been extensively included in GF dough preparations.

The combined presence in the HPMC molecule structure of both hydrophilic and hydrophobic residues along with the ability of binding water molecules in a cold system and release them during heating make these class of hydrocolloids a very versatile food additive in bread making. For instance, such properties can conferee to rice doughs a pseudo-plastic behavior and similar rheological properties to GC doughs (Sabanis & Tzia, 2011; Sivaramakrishnan et al., 2004). Particularly, a recent review described the importance of HPMC employment in GF bread making in order to stabilize those GF systems containing other water binding ingredients such as beta-glucans from oats, rice and soybean brans and flours from other cereals, such as buckwheat and quinoa (Foschia et al., 2016).

Interestingly, the HPMCs have several physicochemical properties that differently characterize their employment in food production. These various physical-chemical features have been neglected so far, leaving a wide margin of opportunity for the research in GF bread making applications.

Nutritional aspects of cereal-based GF products and future possible innovation

Over the years, the research on GF products has mostly followed the technological need of overcoming the lack of a gluten-like network. Based on this research, a combination of refined flours from rice and corn, starches obtained from potatoes or cassava and hydrocolloids mixed with other additives, such as emulsifiers and enzymes, is now considered the best formula to obtain cereal-based GF products (Rosell & Matos, 2015). Conversely, the sensory and the nutritional aspects have not been sufficiently considered (Foschia et al., 2016).

As reported by Rosell & Matos (2015), the various cereal-based GF products currently marketed are often characterized by a bland and an artificial flavor, which may affect the palatability and the consumer acceptance

Furthermore, as mentioned in the previous section, various concerns relative to the high content of refined starchy ingredients in GF cereal products have emerged. Firstly, an increase glycemic index observed in several products due to the presence of rapidly digestible carbohydrates (Pellegrini & Agostoni, 2015; Witczak, Ziobro, Juszczak, & Korus, 2016). But also the scarce contribution to the daily recommended intake of dietary fibers, vitamins and minerals such as B-vitamins, iron and zinc (Foschia et al., 2016). On the other hand, an additional amount of shortenings mainly containing saturated fat, and salt (sodium) are used to improve palatability and tastefulness (Miranda et al., 2014; Tricia Thompson, 2009).

Today the consumers' demand for healthy and nutritious foods is increasing. People seek fresh, natural and minimally processed foods enriched with beneficial ingredients that help to fight diseases and promote good health (Nielsen, 2015). This trend also drives the celiac consumers, confirming the importance of the research and the development of highly acceptable and nutritious cereal-based GF products to fulfil and satisfy the needs of individuals with CD (Anton & Artfield, 2008; Mir, Shah, Naik, & Zargar, 2016).

Interestingly, beside individuals with CD, there is a growing demand for GF products by non-celiac people who believe the gluten exclusion is a good health practice.

Such growing demand of GF products, especially those with high nutritional quality, has not gone unnoticed by food producers, interested in gaining new customer segments (Drabińska, Zieliński, & Krupa-Kozak, 2016). Consequently, a wide range of nutritionally valued raw materials have been proposed to enrich the common formulation of the cereal-based GF products. Among them, non-traditional flours from minor cereals and/or pseudocereals, including buckwheat, quinoa, teff, amaranth in GF products has been added to improve the content of macro and micronutrients of products such as GF bread and pasta (Alvarez-Jubete, Arendt, & Gallagher, 2010). Minor cereals and pseudocereals, *i.e.* quinoa and amaranth, have a good content of protein, whereas buckwheat has a high amount of B-vitamins (Alvarez-Jubete, Arendt, & Gallagher, 2009).

The inclusion of several oligo and polysaccharides from plant foods, such as inulin-type fructans, xylan and exopolysaccharides (EPS) from bacterial sources, is currently considered as a strategy to rise the content in dietary fibers (Capriles, dos Santos, & Arêas, 2016; Galle & Arendt, 2014).

GF breads and pasta formulations have also been enriched with flours from legumes (Foschia, Horstmann, Arendt, & Zannini, 2017), which provide valuable sources of protein, vitamins, minerals, and non-starch complex carbohydrates, such as dietary fibers (Wood, Chibbar, Ambigaipalan, & Hoover, 2010).

The seeds of the species *Salvia hispanica L.* commonly known as chia, have become popular in recent years in the health foods market. These seeds are an important staple food of middle-Americans populations since the pre-Columbian times. In particular the roasted seeds were eaten and the oil was used for the therapeutic proprieties (Reyes-Caudillo, Tecante, & Valdivia-López, 2008).

Nowadays, chia seeds have been recognized as important sources of proteins, antioxidants and dietary fibers by food researchers and dietitians. In this sense, in order to improve the nutritional profile and the technological features of GF doughs and batters, the inclusion of chia seeds as flour or as exuded mucilage is a growing popular strategy (O'Shea, Arendt, & Gallagher, 2014; Steffolani, de la Hera, Pérez, & Gómez, 2014).

Ancient techniques such as the sourdough fermentation have been introduced in the industrial manufacture process in the last few years, as described by Arendt et al. (2007). In particular, sourdough consists of a mixture of water and flours from a single or multiple GF cereals usually buckwheat, sorghum, quinoa and so on, fermented by yeast and lactic acid bacteria.

It has been reported that sourdough is able to improve the texture, but also the flavor and shelf-life of cereal-based GF products (Zannini, Pontonio, Waters, & Arendt, 2012).

As a result of the increased use of ingredients in cereal-based GF preparations and, in turn, of several possible combinations resulting, there is an increased nutritional variability among the GF products today marketed (Capriles & Arêas, 2014; Mir et al., 2016). Therefore, nowadays it may be difficult to draw a conclusion on the overall nutritional quality of cereal-based GF products, also in comparison with the GC counterparts. In view of this, the strategies adopted to evaluate the nutritional quality of GF products may need to be rethought.

In fact, to date the widespread approach to evaluate the nutritional quality of GF products is limited to the only analysis of the content of macro and micronutrients, performed by collecting information from the nutritional facts. An example of this kind of approach comes from the recent publication of

Missbach et al. (2015) who built an extended composition database of macro and micronutrient amounts of a variety of Austrian GF product.
However, it may be important to have in mind that ingredients such as sourdough, minor cereals and pseudocereals flours may have an added value that goes beyond the influence on the amount of macro and micronutrients in the final product. For instance, by the inclusion of sourdough the consequent ameliorated aroma of GF bread may lead to salt or fat reduction (Moroni, Dal Bello, & Arendt, 2009). A possible solution might be the application of a scoring system that take into account the overall contribution of these type of ingredients to the food item nutritional profile by assigning them a representative value.

Importance of dietary fiber intake for individuals with CD

In the last decades, dietary fibers have been gaining importance due to their protective effect on cardiovascular health, diabetes, obesity, intestinal motility and cancer (Minihane et al., 2015). However, although the positive effect of dietary fibers on health, recent data from the US National Health and Nutrition Examination Survey (NHANES) and European Prospective Investigation into Cancer and Nutrition (EPIC) highlighted their low consumption (Murphy et al., 2012).
Along with the protective effect on non-communicable diseases, the role of various dietary fibers in shaping the large intestinal microbiota composition by acting as prebiotics is being evaluated. Prebiotics are food constituents that pass not digested through the small intestine. When prebiotics arrive in the large intestine they can preferentially induce the metabolism and the growth of some beneficial bacterial species called commensal bacteria (Roberfroid, 2007b). These commensal bacteria can modify the gut environment avoiding the proliferation of those bacterial species that are able to activate the immune response and the inflammatory cascade. Therefore, it has been suggested that prebiotics fibers may help to maintain or restore a healthy gut environment.
Among prebiotic fibers the inulin-type fructans, or simply inulin, have risen the interest for the ability of influencing the growth of the commensal bacteria of the large intestine in humans.
Briefly, inulin are mixtures of oligo and polysaccharides of fructose and glucose extracted from the edible part of several fruits and vegetables, mainly chicory roots (Roberfroid, 2007). Inulin may act as hydrocolloids by retaining water and modifying the viscoelastic properties of doughs (Drabińska et al., 2016; Foschia et al., 2016). In addition, inulin has been long used as fat and sugar replacers in both cereal and dairy products. However, recently the interest over inulin has moved towards the inclusion in cereal-based formulations to increase the dietary fibers content of baked products, especially of GF bread (Mensink, Frijlink, Van Der Voort Maarschalk, & Hinrichs, 2015).

In-vitro and *in-vivo* studies evidenced the ability of inulin to influence in particular the bifidobacteria species of the human large intestine, which are thought to exert a powerful antipathogenic activity. In fact, through the production of metabolites as the short chain fatty acids they are responsible for the colonization resistance to pathogenic species in the human gut (Kolida & Gibson, 2007).

However, the inclusion of inulin in bakery products still presents some issues, such as the hydrolysis of the central chain of the inulin molecule during the production process, which leads to a reduction in its final concentration. Interestingly, the causes of this degradation are not fully understood, thus offering several researches prospective.

In recent years, other dietary fibers such as the EPS have raised the interest of the researcher interested to their possible technological applications as well as for their observed prebiotic activity (Ruas-Madiedo, Hugenholtz, & Zoon, 2002; Zannini, Waters, Coffey, & Arendt, 2016).

EPS are dietary fibers which consist of a heterogeneous group of polysaccharides with high molecular weight and a degree of polymerization. These polysaccharides are formed by a complex chain of repeated units of glucose (dextrans) or fructose (levans). EPS are naturally produced by lactic acid bacteria commonly used as starter culture in several fermented foods, such as yogurt and beverages (Zannini et al., 2016). These polysaccharides gained importance in food industry due to their ability to bind water, interacting with proteins and increase the viscosity of milk and fruit juices. In bread making the interest over EPS has increased because they are produced by the lactic acid microbiota of the sourdough. In particular, the ability to act as hydrocolloids improving volume, texture, flavor and shelf life of final product have been observed and appreciated by food manufacturers. Their possible application in GF bread making as co-adjuvants of the other hydrocolloids was reviewed by Moroni et al. (2009) and currently constitutes an open line of research.

Concerning the possible application of EPS as prebiotic fibers to be included in food preparations, several studies attempted to observe their effect on the growth of several microbiological species considered beneficial for the human host. Interestingly, EPS showed the ability to act as fermentable substrates for microorganisms in the gut human environment, modifying the interaction among the intestinal populations (Hongpattarakere, Cherntong, Wichienchot, Kolida, & Rastall, 2012; Rios-covian et al., 2016; Salazar, Gueimonde, Hernández-Barranco, Ruas-Madiedo, & De Los Reyes-Gavilán, 2008).

It is worth noticing that in the latest studies on environmental triggers of CD it has been suggested a possible role of the intestinal microbiota in the well-being of individuals with CD (Maria C. Cenit, Codoñer-Franch, & Sanz, 2016; de Sousa Moraes, Grzeskowiak, de Sales Teixeira, & do Carmo Gouveia Peluzio, 2014; Marasco et al., 2015). In fact, an altered intestinal bacterial composition may determine a chronic low grade inflammation of the intestinal mucosa with possible negative effects on the intestinal permeability and the tolerance toward the food antigens, as gluten (Sanz, 2015; Wacklin et al., 2013).

With this in mind, the consumption of foods containing prebiotic fibers by individuals with CD might constitute a potential contribution to managing the intestinal manifestations of the celiac conditions along with ameliorating the nutritional and technological characteristics of these products.

1.4 Research purpose of this thesis

GF diet requires GC foods to be replaced with other foods obtained with GF ingredients. However, that is not all.

On one hand, there is the production of GF products with a good quality, in terms of nutritional value and consumer acceptability, which relies on an appropriate selection of nutritious ingredients and a crucial management of the stability of the GF system. The latter is certainly influenced by the presence of ingredients with different physical-chemical properties, e.g., the water binding ability of dietary fibers rich ingredients. In this scenario, it is pivotal to deepen the role of important food additives known for their technological properties as the HPMC and/or for their nutritional importance, such as chia seeds, inulin-type fructans and EPS, in the production of healthy, nutritious and high-quality GF foods.

On the other hand, not only the compliance with the GF diet but also the dietary choices of individuals with CD deserve the attention of physicians and other healthcare professionals involved in the follow-up of these patients. Indeed, except for few individuals with CD with comorbidities, such as type-1 diabetes, for the majority of individuals with CD there is not a short-term risk for health by following an unbalanced diet. However, as for the general population, it is the long-term exposure to unbalanced dietary choices that can prefigure an increased risk of non-communicable diseases.

Therefore, it will be interesting to explore the different difficulties and strategies regarding the quality of GF products and diet. In this view, this thesis will illustrate four different research actions aimed at:

- Proposing a simple tool to describe the nutritional profile of packaged GF bakery products in the perspective of leading their nutritional enrichment and comparing their nutritional quality to that of GC counterparts.

  Chapter 2 - Designing a score-based method for the evaluation of the nutritional quality of the gluten-free bakery products and their gluten-containing counterparts.

- Deepening the functional relationship between two of the major players in the stability of a GF system, such as polysaccharide hydrocolloids and water.

  Chapter 3 - Understanding the role of hydrocolloids viscosity and hydration in developing gluten-free bread.

- Addressing the role of Chia seeds and/or exuded mucilage as potential thickening agent and nutritional enhancer in production of GF pasta with similar characteristics to wheat pasta.

Chapter 4 - Gluten-free pasta incorporating chia (Salvia hispanica L.) as thickening agent: An approach to naturally improve the nutritional profile and the in vitro carbohydrate digestibility.

- Studying the dietary habits of a group of Italian individuals with CD with the aim of evaluating their adherence to a food pattern recognized for its protective role on the risk of major non-communicable diseases.
  Chapter 5 - Are the dietary habits of treated individuals with celiac disease adherent to a Mediterranean diet?

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Chapter 2

Designing a score-based method for the evaluation of the nutritional quality of the gluten-free bakery products and their gluten-containing counterparts.

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ABSTRACT

Gluten-free (GF) products are consumed both by individuals with celiac disease (CD) and by an increasing number of people with no specific medical needs. Although the technological quality of GF products has been recently improved, their nutritional quality is still scarcely addressed. Moreover, the few published studies report conflicting results, mostly because the information from product nutrition facts is the only considered factor.

The aim of the present study was to develop a score-based method for the nutritional evaluation of 134 packaged Italian GF bakery products and compare it with that of 162 matched gluten-containing (GC) food items. The score included the information from the nutrition facts and the presence/absence of some nutritionally relevant components in the ingredients list. Results indicated an overall low nutritional quality of the considered GF bakery products. Additionally, with the sole exception of GF bread substitutes, there was no difference in nutritional quality between GF and equivalent GC bakery products. Future research and development of GF bakery products may take advantage of this score-based method, as it may represent an easy approach to evaluate their nutritional quality. The present findings do not justify the consumption of packaged GF bakery products by people without any specific medical needs.

Keywords

Nutritional quality, gluten-free bakery products, score-based method, celiac disease.

Abbreviations

CD: Celiac disease

GC: Gluten containing

GF: Gluten-free

LAB: Lactic acid bacteria

1. INTRODUCTION

The total exclusion from the diet of foods containing gluten is the only possible treatment for celiac disease (CD), an autoimmune disorder sustained by an inappropriate response to gluten ingestion in genetic predisposed individuals [1]. A gluten-free (GF) diet includes naturally GF foods, such as vegetables, fruits and meat, and GF products developed to substitute the traditional cereal-based foods.

It has been estimated that at least 5% of the world population needs to follow a GF diet for medical purposes [2], although a specific medical need is not an essential reason to follow it. Furthermore, the GF diet has recently become a kind of cultural phenomenon involving the search for foods free of one or more ingredients that are supposed to be unnatural or unhealthy [3, 4]. Consequently, the GF market has recently seen a remarkable growth, with sales of GF foods increased about by 136% between the 2013 and 2015 in the US, reaching a total value of around $11 billion [5]. In Europe, the latest economic reports foresee a regular growth rate of about 10% until 2019 [2].

Owing to the growing interest in GF products, their formulation and production processes have been recently put under the spotlight, with a peculiar attention towards GF bakery products. However, all these efforts in GF product development and/or improvement have been mainly focused on the technological and sensory aspects, leaving the nutritional quality very poorly addressed [6].

To overcome the technological constraints associated to the absence of gluten, and therefore improve the texture and the sensory characteristics of GF products, various food additives and co-texturizers are applied [7]. These ingredients obviously modify the nutritional composition of GF products and, in turn, may affect their nutritional quality.

Despite a growing popular perception that GF products are healthier than the gluten-containing (GC) counterparts, their real nutritional quality is still far to be conclusively defined, and a huge number of variables are involved in its definition. Actually, a limited number of conflicting studies have assessed the nutritional quality of GF products and compared it to that of their GC counterparts. Some authors [8, 9] have reported a higher content of total and saturated fat in GF products, whereas others [1, 10] have found no differences between the two types of product in terms of such nutrients. In addition, inconsistent results about the content of dietary fiber have been reported [1, 9]. Such discrepancies in nutritional quality definition of GF products may also be attributable to the high variation of GF formulations and/or to a possible small interpretative ability of the methods used to measure the nutritional quality.

To try to partially address this issue, and referring to the Italian market of GF products, we have developed a score-based method in order to assess the quality of packaged GF products and to compare with that of similar GC counterparts. The focus of this work is on the bakery products as they represent staple foods largely consumed and important sources of nutrients for the general population.

2. MATERIALS AND METHODS

2.1 Selection of the products.

According to the latest trends in sales of the Italian food market (2015), kindly provided by Dr. Schär GmbH/Srl, packaged products from the most representative Italian brands (almost 60% of the market sales) producing GC and/or GF bakery foods were selected for the present study. GF bakery products and their GC counterparts were grouped into four food categories: bread, bread substitutes, cookies and breakfast pastries. The list of the type of products analyzed in each food categories is reported in Table 1S. Information about the nutritional composition and ingredients was directly collected on both the food manufacturer's website and the product pack.

2.1 Design and application of a score-based method.

We developed the score-based method by considering two groups of parameters: i) amount of specific macronutrients and ii) nutritional quality of some ingredients in the food formulation.
The first group of parameters was quantitative, and included total and saturated fat, sodium, fiber and sugar. Their reference amount was selected according to the *EU regulation on nutrition and health claims made on foods* (Regulation (EC) No 1924/2006 – Annex "Nutrition claims and conditions applying to them"). The quantification was based on the nutrition facts information available on the food pack label, and the relative amount of such parameters was scored with points from 0 to 2, as described in Table 1. The overall sum may reach up to 7 points.
The second component of the score was qualitative, and designed to emphasize the presence or absence of specific ingredients in determining the overall nutritional quality of the considered products. The qualitative parameters were selected according to the recent proposed strategies to improve the nutritional quality of the GF bakery products [12–14]. In particular, as described in Table 2, the presence/absence (yes/no) of the following ingredients was evaluated: i) starch as first or principal ingredient; ii) wholegrain flours; iii) sourdough (only as a leavening agent); iv) flour from legumes; v) other flours, from minor cereal and/or pseudocereals (*i.e.* buckwheat, quinoa, amaranth and sorghum, used as alternative to wheat or traditional GF cereal) [15]; vi) fructose; vii) emulsifiers (mono and diglycerides of fatty acids). The score for each product was obtained by summing the points assigned to the amount of specific nutrients (quantitative parameters) and the points resulted from the qualitative parameters. As the number of qualitative parameters used to describe each food

category was different, the maximum score was different among food categories. In particular, for bread and bread substitutes the score ranged from 0 to 13 points, for breakfast pastries from 0 to 12 points, and for cookies from 0 to 11 points.

Table 1 Considered information from nutritional facts of products and points assignment for the quantitative part of the score calculation[a].

Parameters	*Zero points*	*One point*	*Two points*
Total fat (g/100 g)	> 3	< 3	
Saturated fat (g/100 g)	> 1.5	< 1.5	
Sodium (g/100 g)	> 0.4	< 0.4	< 0.12
Fibre (g/100 g)	< 3	> 3	
Sugar (g/100 g)	> 5	< 5	< 0.05

[a]According to the limits stated in the Regulation (EC) No 1924/2006 – *Annex "Nutrition claims and conditions applying to them"*

Table 2 Considered nutritionally relevant ingredients and points assignment for the qualitative part of the score calculation[a].

Parameters	*Zero points*	*One point*
Starch as first ingredient	Yes	No
Wholegrain flours	No	Yes
Sourdough[1]	No	Yes
Flour from legumes	No	Yes
Other flours[2]	No	Yes
Fructose[3]	Yes	No
Emulsifiers[4]	Yes	No

[a]Points were assigned according to the presence/absence (yes/no) of the ingredients.
[1]only bread; [2]GF ingredients different from rice and corn, such as buckwheat, quinoa, sorghum, etc. and GC cereals different from wheat, such as rye and barley; [3]in the form of corn syrup in cookies and breakfast pastries; [4]mono- and diglycerides of fatty acids.

2.3 Statistical analyses.

Shapiro-Wilk test was used to evaluate the normality of distributions. The score obtained for the GF bakery products was compared to that obtained for the GC counterparts by means of the Mann-Whitney test. To determine whether the score method misclassified the considered products, a further evaluation by means of the Mann-Whitney test based only on the quantitative parameters was performed.

All data analyses were performed by using IBM SPSS® Statistics software 22.0 (IBM Corp., Chicago, IL). Significance was accepted at $p < 0.05$.

3. RESULTS AND DISCUSSION

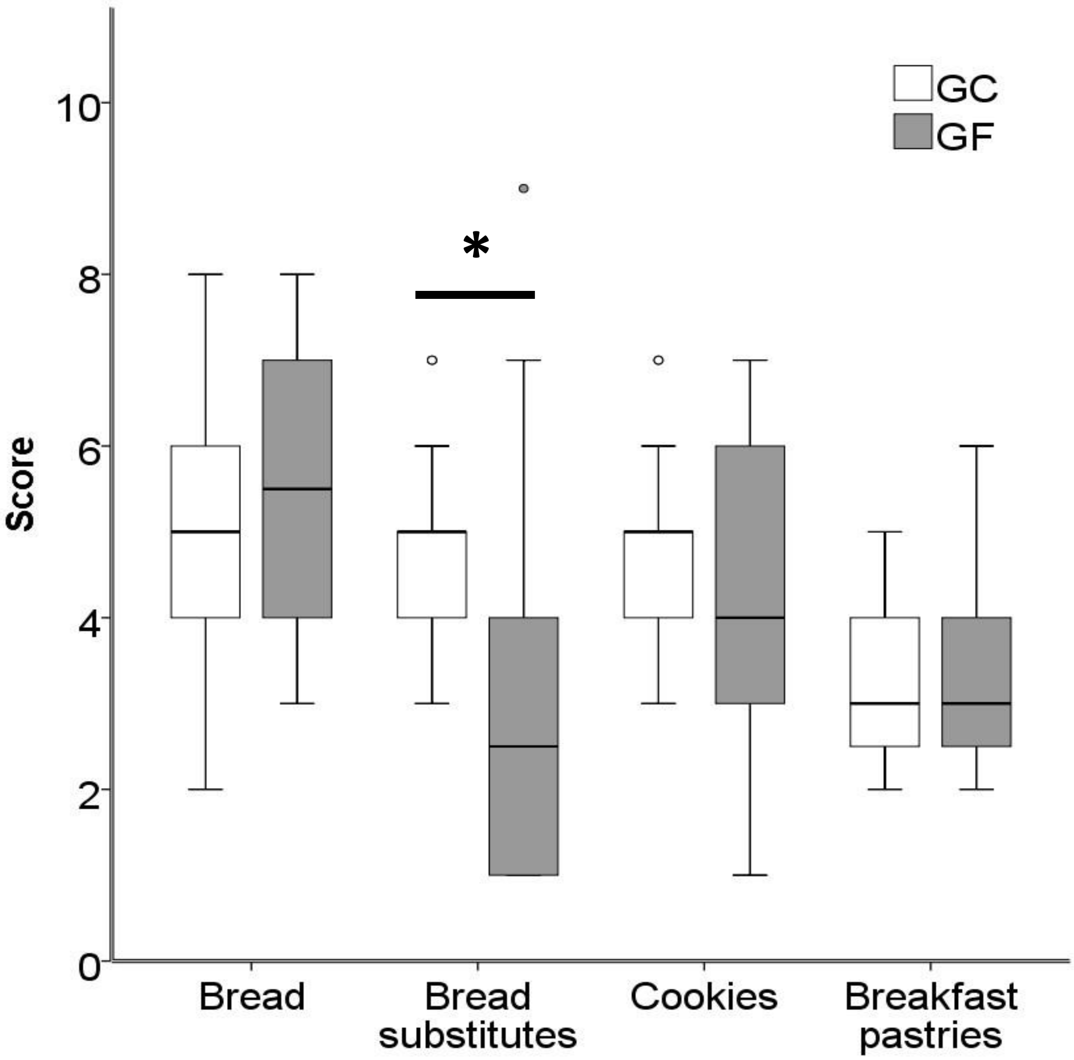
The evaluation of the nutritional quality of GF products has been mainly based on the information retrievable on nutrition facts [9, 10, 16]. Nevertheless, the nutritional quality of a bakery product cannot be only ascribed to its macro- and micro-nutrient content.

For instance, the inclusion of flours rich in dietary fiber in the formulation of bread products, e.g., those obtained from amaranth, quinoa or buckwheat, is a common practice [17]. However, these flours may influence more than the only content of dietary fiber. Indeed, they allow to partially replace ingredients such as starch from potato or cassava and refined flours present in the formulation, thus improving the content of several nutrients scarcely contained in GF bakery products, e.g., proteins, various vitamins and minerals [18]. Accordingly, the added value of these ingredients goes beyond the simple influence on one nutrient.

In this study, a total of 134 Italian packaged GF and 162 GC bakery products, grouped into four food categories, were evaluated using a nutritional quality score-method. This score considered not only the information from nutrition facts, but also the contribution of some nutritionally relevant components in the ingredients list.

Applying this score, an averagely low nutritional quality of the considered GF bakery products emerged, as observed in Figure 1. Interestingly, GF bread, cookies and breakfast pastries scored relatively close to their GC counterparts. The only clear exception were GF bread substitutes, which showed a significantly lower nutritional quality when compared to their GC counterparts ($p = 0.001$).

Fig. 1 Box-plot graphs showing the score of GF products compared to that of the GC counterparts. For bread and bread substitutes, the score ranged from 0 to 13 points, for breakfast pastries from 0 to 12 points and for cookies from 0 to 11 points. (*) indicates a significant difference, Mann-Whitney, $p = 0.001$.



The findings of the present study are in agreement with those reported by Wu et al., [1], who compared the nutritional quality of several Australian packaged GF products, across ten food categories, to their matched GC counterparts. The nutritional quality of GF products was based on a descriptive score, namely the "Health Star Rating" (HSR) system. Indeed, the HSR system is a combination of some baseline points, taking into account the amount of saturated fat, total sugar and sodium, and of several points attributed by the presence of specific food components, including fruit, nuts, vegetables, legumes, and the content of protein and dietary fiber. Authors evidenced that the GF bakery products in several food categories, such as bread, cakes, and cookies, were not significantly different in their nutritional quality when compared to GC similar items.

Our results are instead in disagreement with those of Miranda et al. [9] and of Kulai and Rashid [16], who considered only the nutrient content. The first study evidenced a significantly better nutritional profile of GC in comparison to GF products in terms of the content of energy, saturated, and total fats. In the study of Kulai and Rashid [16], the GC breads showed better nutritional value than GF substitutes, as the latter were significantly higher in total fat and lower in protein.

Despite the low nutritional quality portrayed by the score, some attempts at improving the nutritional quality of GF bakery products emerged (Table 3) from our observations.

Starch is one of the most relevant ingredients deeply affecting nutritional quality of GF bakery products. Due to its bland taste, starch presence as first or main ingredient entails salt and lipid addition to GF bakery products in order to enhance their low palatability [12]. Table 3 shows that in 42% of considered GF bread formulations starch was not the first or principal ingredient.

The main strategy for reducing starch content in bakery products is its partial substitution with flour obtained from nutritionally valued minor cereals and pseudocereals, especially in GF bread making [15, 19]. Among these alternative ingredients, quinoa, buckwheat, and sorghum have attracted attention because of their very interesting nutritional composition, providing relevant amounts of dietary fiber, B-vitamins and iron [18]. Interestingly, our results confirmed that this enrichment trend involves several GF breads, as 79% of the evaluated products contained flours from minor cereals and/or pseudocereals, and the 88% could be labelled as "source of fiber" according to the Regulation (EC) No 1924/2006 (Table 3). These data seem to disagree with the general belief that GF bakery products scarcely contribute to the daily intake of dietary fiber [20, 21].

In the last few years, the sourdough fermentation has been introduced in the production of industrial GF bread. In this case, the sourdough is composed of a wide range of GF flours (rice, corn, buckwheat, etc.) and water, and is fermented by yeasts and lactic acid bacteria (LAB) [22]. LAB produce long-chain polysaccharides that may act as a co-adjuvant of the common hydrocolloids used in GF bread making [22]. In view of this, the sourdough employment seems to fulfil more a technological purpose rather than a nutritional enhancement. However, it is worth underlining also that these long-chain polysaccharides contribute to the daily intake of dietary fiber, and may behave as prebiotics [23]. In fact, some studies have shown that these polysaccharides may be fermented by the intestinal microbiota and in turn modulate the immune response [24, 25].

Considering our results, sourdough was present in 54% of GF breads formulations compared to 21% of the GC breads.

In contrast with some improvements emerged in GF bread production, GF bread substitutes resulted relatively disappointing. Starch was not the first ingredient in only the 23% of GF bread substitutes and no wholegrain flour was included in their formulations (Table 3). Flours obtained from other cereals were included in 27% of GF bread substitutes, against the 47% of the GC similar products. As a consequence, only the 46% of GF bread substitutes could be labelled as "source of fiber", with respect to the 88% of their GC counterparts (Table 3). To date, bread substitutes represent a substantial part of the sales of GF bakery products [26] and they are often consumed as a snack or an alternative to bread by individuals with CD [27]. For this reason, great care should be taken to improve their nutritional composition.

Cookies and breakfast pastries, in general, are driven, in their formulation, by different marketing needs. Their content of sugar and total fat – but also the quality of these fats – is functional to ensure their specific texture, their palatability and, as a consequence, consumer acceptability [28]. Therefore, we did not expect GF cookies and breakfast pastries to be low in sugar, total and saturated fat. However, considering the positive results reported by some studies aiming to improve the nutritional value of these GF products [29, 30], we were expecting to identify more products containing whole grain flours and/or flours from minor cereals and pseudocereals at least.

Table 3 Percentages of products, divided into food categories, matching the conditions[1] used to calculate the score.

Food category		Low fat	Low saturated fat	Low sodium	Source of fiber	Sugar free	Low sugar	Starch as first ingredient	Wholegrain flours	Sourdough	Flour from legumes	Other flours	Fructose	Emulsifiers
		%	%	%	%	%	%	%	%	%	%	%	%	%
Bread	GC (n= 34)	9	62	0	76	0	50	0	38	21	12	47	n.u	15
	GF (n= 24)	4	67	0	88	0	88	58	4	54	67	79	n.u	63
Bread substitutes	GC (n= 49)	2	16	2	88	0	96	0	16	n.u	0	47	n.u	8
	GF (n= 26)	8	12	0	46	12	62	77	0	n.u	19	27	n.u	59
Cookies	GC (n= 43)	0	5	28	56	0	9	2	0	n.u	7	n.u	28	2
	GF (n= 53)	0	0	30	42	0	0	42	0	n.u	49	n.u	6	28
Breakfast pastries	GC (n= 36)	3	8	8	17	0	6	0	0	n.u	0	n.u	53	89
	GF (n= 32)	0	0	3	25	0	6	38	0	n.u	28	n.u	19	63

[1]Regulation (EC) No 1924/2006 about nutrition and health claims (quantitative parameters); presence/absence of nutritionally relevant ingredients (qualitative parameters). n.u means that the ingredient is not used.

Among the limitations of this scoring method is its partially qualitative nature. Some parameters may have negatively affected the comparison between GF and GC products, since the score was mainly set-up to evaluate the nutritional quality of the GF bakery products. For instance, flours from legumes are often incorporated in GF bakery products to improve qualitative characteristics, such as viscoelastic functionality of doughs, sensory acceptance and shelf-life [13], but they are barely present in the formulation of GC bakery products. However, although the developed score method was designed for the nutritional evaluation of GF bakery products, the results did not change when the evaluation was based on the sole quantitative parameters (Table 4).

Table 4 Comparison between GF products and GC counterparts based on the quantitative parameters.

		Score Percentiles			
Categories		25th	Median	75th	*p*-value[1]
Bread	GC	1.00	2.00	3.00	0.06
	GF	2.00	3.00	3.00	
Bread substitutes	GC	2.00	2.00	2.00	<0.01
	GF	1.00	1.50	2.00	
Cookies	GC	1.00	2.00	2.00	0.20
	GF	1.00	2.00	2.00	
Breakfast pastries	GC	1.00	1.00	2.00	0.82
	GF	1.00	1.00	2.00	

Statistical analysis was performed by comparing the points obtained by GF and GC bakery products by applying only the quantitative parameters. [1]Mann-Whitney test, $p < 0.05$.

Also in this case, the considered Italian GF bakery products had a low nutritional profile similarly the GC counterparts. The only exception was for the GF bread substitutes, which obtained significantly less points than those of their GC counterparts ($p = 0.005$).

The low nutritional quality of all the considered products (i.e., GF and GC) may be partly explained by the fact that they were packaged food items. In this sense, it is worth to remind that some ingredients used in packaged bakery goods, such as emulsifiers and salt, cannot be completely avoided or reduced due to their role in both the GC and GF baking process [5]. For example, it is quite a challenge to produce sliced bread by lowering salt content below the value established as "low in sodium" by EU Regulation (EC) No 1924/2006 (*i.e.* 0.12 g/100 g of sodium or the equivalent 0.3 g/100 g of salt), without affecting some important quality parameters, such as texture and shelf life [31].

CONCLUSIONS

Based on the results of this study, the nutritional quality of the analyzed Italian GF bakery products resulted low and comparable to that of GC counterparts. Therefore, the present findings do not justify the consumption of packaged GF bakery products instead of the traditional GC ones by people without any specific medical need. Rather, this work suggests that the formulation of these products should be revised in order to improve their nutritional profile. In this view, the developed scoring method could be used to address the future development of such products, as it may represent an easy approach to evaluate the nutritional quality of the GF bakery products. The further integration of the developed score with information about the micronutrients content of the evaluated products would be useful to allow a more comprehensive nutritional evaluation.

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Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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SUPPLEMENTARY MATERIALS

Table S1 Number and type of products included in each food category and sub-category.

Categories	**Sub-categories**	**Gluten-containing products**	**Gluten-free products**	**Total**
Bread (sliced)	White, rustic	12	9	21
	Multigrain and cereals	14	14	28
	Wholegrain	8	1	9
	Total	34	24	58
Bread substitutes	Crackers: plain, rustic, salted, non-salted	21	14	35
	Crackers: wholegrain	9	0	9
	Breadsticks: plain, rustic, salted, non-salted	19	12	31
	Total	49	26	75
Cookies	Plain	10	18	28
	Chocolate	6	13	19
	Filled with milk or chocolate cream	8	10	18
	Wholegrain	5	1	6
	Wafer with milk, chocolate, vanilla cream	14	11	25
	Total	43	53	96
Breakfast pastries	Plum cake: plain, yogurt, chocolate	9	11	20
	Croissant with jam, cream, or chocolate	17	4	21
	Sweet snack with jam, milk, chocolate cream	7	14	21
	Muffin	3	2	5
	Total	36	31	67
	Total	162	134	296

Chapter 3

Understanding the role of hydrocolloids viscosity and hydration in developing gluten-free bread.

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Abstract

To understand the role of hydrocolloids' viscosity in developing gluten-free (GF) bread, a range of hydroxypropylmethylcellulose (HPMC) with similar backbone and percentage of methoxyl and hydroxypropoxyl residues was selected in order to vary the viscosity (100 cP, 4000 cP, 15000 cP) while keeping the main chemical structure. Viscosity of HPMC was used as quantitative independent factor in a multilevel factorial model along with HPMC level (1%, 2%, 3%) and hydration level (90%, 100%, 110%). The model results in 27 formulations based on rice flour. A Mixolab system was uses to analyse the rheological parameters of the GF batters during mixing, cooking and cooling stage. In addition, GF breads were characterized for their quality parameters. Analytical data were fitted to multiple regression equations in order to estimate the dependence of the collected parameters on the quantitative independent factors. Results confirmed the importance of hydration level in determining the viscoelastic behaviour of the GF batter and influencing the rheology characteristics of bread. Moreover, it was possible to underline the role of the HPMC viscosity along with the level of HPMC, both to control the batter consistency and some desirable textural features of GF bread such as crumb hardness, cohesiveness and resilience. Finally, some analytical parameters were used as quality indicators in desirability index calculation, which would represent a GF breads with optimum quality obtained by the inclusion of a 2.2% of HPMC 15000 cP with hydration level to 110%.

Key words: hydrocolloids; viscosity; hydration; gluten-free bread; texture.

1. Introduction

Traditional bakery products manufacturing, i.e. bread, relies on gluten presence in flours from cereals such as wheat, rye and barley. Proteins of gluten when hydrated form a viscous mass that confers to the dough, structure, viscosity, mixing tolerance and gas holding ability during leavening. In addition, the resulting structure encloses flour components such as starch granules and fibres (Gallagher, Gormley, & Arendt, 2004). With this in mind, producing high quality gluten-free (GF) foods such as bread represent a challenge for food manufacturer. In fact, proteins contained in GF cereals, such as rice and maize, do not have the same viscoelastic and structuring properties of gluten (Renzetti & Arendt, 2009). Consequently, doughs are liquid and similar to batters and the bread has several post-baking quality defects, such as dry and crumbly texture and low specific volume.

However, there is scarce information about the main players in developing a network resembling gluten functionality that leads to aerated bread structures. Some studies pointed out the importance of dough or batter consistency after mixing and during heating and cooling of the systems (Matos & Rosell, 2014). Particularly, it has been found a strong relationship between the crumb hardness with consistency during mixing and during cooling, stating the importance of hydration and cooling consistency (Matos & Rosell, 2013). In a GF system, hydration contribute to define the rheological properties of GF dough and bread. GF flour components such as starch granules can form a continuous viscous phase when water is added (Crockett & Vodovotz, 2011). In particular, although it depends on the type of GF raw material, it was highlighted that by adding water it is possible to control several aspects of GF dough and bread such as loaf specific volume and crumb hardness (Rózyło et al., 2015). In absence of structural components, the viscous phase due to starch relies only on fragile intermolecular bonds that weaken the stability of a GF system. Consequently, the viscosity is low and the flow ability of flour components such as starch remains high, thus explaining the low mixing tolerance and poor extensional properties of GF doughs (Lazaridou, Duta, Papageorgiou, Belc, & Biliaderis, 2007).

It has been largely known that hydrocolloids act as structuring agents in GF matrixes. In fact, through hydrocolloids it is possible to control the water binding and produce a gel network structure that serves to increase the dough intermolecular viscosity stabilizing the GF system (Anton & Artfield, 2008; Houben, Höchstötter, & Becker, 2012). For that purpose, different hydrocolloids have been reported, like xanthan gum, guar gum, cellulose derivatives, pectins, and so on (Houben et al., 2012). Nevertheless, cellulose derivatives are among the most common hydrocolloids used to replace gluten. In GF bread making, the hydrocolloids in general and hydroxypropylmethylcellulose (HPMC) in particular, have been very effective in solving some of the GF bread quality issues. In fact, it have been observed that hydrophilic bonds formed by HPMC can be effective on water absorption and on flow ability of rice flour components reducing the repulsive forces, supporting the homogeneity and the stability of the GF systems (Rosell, Yokoyama et al. 2011); Sivaramakrishnan, Senge, & Chattopadhyay, 2004). In addition, hydrophobic groups of HPMC can help to form a network structure that wrap-around the flour components of GF batters (Crockett et al., 2011; Lazaridou et al., 2007) improving development, gas retention during leavening and final specific volume of breads (Mancebo, San Miguel, Martínez, & Gómez, 2015).

Currently, studies that aim to evaluate the mechanism of interaction between HPMC and water in a GF system have been mainly focused on the hydration and HPMC level to be used (McCarthy, Gallagher, Gormley, Schober, & Arendt, 2005; Sabanis & Tzia, 2011). Those have pointed out that the functionality of HPMC is highly dependent of the type of raw material used, the presence of other additives and the percentage of methoxyl groups of the HPMC (Crockett et al., 2011; Hager & Arendt, 2013). Reaching this point, it must be stressed that loaf volume increment and crumb hardness reduction are likely linked to a certain limit of HPMC and water level (Mancebo et al., 2015; Sabanis & Tzia, 2011).

Finally, it is worth noticing that the term HPMC comprises a range of compounds that provides different viscosities. However, very scarce information exists exploring HPMC viscosity functionality in GF mixtures. With this in mind, this study aim to assess the individual and combined effects of fundamental factors such as HPMC viscosity, HPMC level and hydration level in a rice based GF system by the means of a an experimental design based on a multilevel factorial model (Rosell, Santos, & Collar, 2010). Rice flour was chosen because of its many unique attributes that makes this cereal suitable for GF bread making (Phongthai, D'Amico, Schoenlechner, & Rawdkuen, 2016).

2. Materials and Methods

2.1 Materials

White rice flour was purchased from Harinera La Meta S.A (Lleida, Spain). HPMC, 19-24 % of methoxyl and 7-12 % of hydroxypropoxyl, with different viscosities were generously donated by Dow Pharma & Food Solutions (La Plaine Saint Denis, France). Specifically, METHOCEL™ K99 (viscosity 100 cP); METHOCEL™ K4M (viscosity 4000 cP); METHOCEL™ K15M (viscosity 15000 cP). Compressed yeast from DHW Europa (Deutsche Hefewerke GmbH, Germany) was purchased. The rest of ingredients were acquired in the local market.

Table 1 – Multilevel factorial design for sampling.

Runs	HPMC viscosity	HPMC level	Hydration level
1	-1	-1	-1
2	0	-1	-1
3	1	-1	-1
4	-1	0	-1
5	0	0	-1
6	1	0	-1
7	-1	1	-1
8	0	1	-1
9	1	1	-1
10	-1	-1	0
11	0	-1	0
12	1	-1	0
13	-1	0	0
14	0	0	0
15	1	0	0
16	-1	1	0
17	0	1	0
18	1	1	0
19	-1	-1	1
20	0	-1	1
21	1	-1	1
22	-1	0	1
23	0	0	1
24	1	0	1
25	-1	1	1
26	0	1	1
27	1	1	1

-1, 0, 1 indicate the code levels of design factors: HPMC viscosity (100 cP, 4000 cP, 15000 cP); HPMC level (1%, 2%, 3%); Hydration level (90%, 100%, 110%).

2.2 Methods

2.2.1 Experimental design

A multilevel factorial model was used to investigate the role of HPMC viscosity, HPMC level and hydration level, as quantitative independent factors, on rheological behaviour of a series of GF batters and on the technological quality of the resulted GF breads. The quantitative independent factors were tested at three levels (-1, 0, +1). The model resulted in 27 different combinations as reported in Table 1.

2.2.2 Rheological behaviour of the GF batters

Rheological behaviour of GF batters was characterised with the Mixolab® (Chopin, Villeneuve la Garenne, France), collecting information about rheological properties of GF batter during mixing, cooking and cooling (Matos and Rosell, 2013). Settings used in the Mixolab® followed the Chopin+ protocol defined by the device supplier, with minor modifications. Briefly, 100 g of GF batter was prepared in the mixing bowl of the Mixolab® by pouring firstly rice flour, salt (1.5%, f. b.) and the HPMC. The test started when the mixing bowl reached 30 °C with a constant mixing speed of 80 rpm. Water (previously heated at 30 °C) was added according to Table 1 and mixed for 8 min. Then batter was heated from 30 ºC to 90 °C over a period of 15 min at the rate of 4 °C/min. Batter was kept at 90 °C for 7 min then cooled to 50 °C at the rate of 4 °C/min and finally held at 50 °C for 5 min (Rosell et al. 2010).

According to previous studies (Matos & Rosell, 2013; Rosell et al., 2010), the following Mixolab® parameters that characterise the consistency of the batter during mixing, cooking and cooling phases were collected: initial consistency or first peak (C1, Nm), minimum torque (C2, Nm), peak torque (C3, Nm), minimum torque during the heating period (C4, Nm) and the torque obtained after cooling at 50 °C (C5, Nm). Derived parameters such as breakdown (C4-C3, Nm), cooling setback (C5–C4, Nm) and total power for the assay (Total PA, kWh/kg) were also recorded.

2.2.3 Bread making process

GF breads were made according to the same combinations and recipes designed for the Mixolab® analysis but including the yeast. Consequently, 1 kg of batter was prepared by mixing flour, salt, HPMC, water at 20 °C and compressed yeast (3%, f.b) for 8 min in a kneader (MAHOT Fork mixers, Montaigu, France). A pastry bag was used to fill metallic pans (width 9 cm; depth 6 cm; height 5 cm) with 100 g of batter. Pans were leavened in a proofing chamber at 30 °C for 30 min and then baked in an electric oven (F106, FM Industrial, Córdoba, Spain) at 185 °C for 40 min. Breads were left cooling down at room temperature until reaching 25 °C in the centre of the loaf before slicing them into 10-mm thickness.

2.2.4 Quality assessment of the GF breads

Instrumental quality parameters were determined as previously described Matos & Rosell (2013), including moisture content (ICC no. 101/1, 1976), crumb texture profile analysis (TPA), crumb image analysis and colour. TPA was performed using the TAXT-Plus Texture Analyses (Stable Micro Systems Ltd., Godalming, UK) equipped with a 5 kg load cell and a 25-mm aluminium cylindrical probe. During the test, the probe double compress the centre of the crumb (the crust was removed) up to 50% strain (penetration of its original height) at a crosshead speed of 1 mm/s and 30 s gap between compressions, providing insight into how samples behave when chewed (Rosell, Santos, Sanz Penella, & Haros, 2009). Quality parameters, such as hardness (g), cohesiveness, adhesiveness, resilience and springiness were collected. Data acquired were the average value of ten replicates.
High-resolution images (600 dpi) of three slices from each run were captured using a high-resolution scanner (HP Scanjet G3110) and then analysed to obtain the morphogeometry and crumb cell characteristics by an image analysis program (ImageJ, NIH, USA). Samples were modified by increasing the contrast between cells and crumb, then “otsu” algorithm was used to define the threshold of the image, according to Gonzales-Barron & Butler (2006). The representative parameter selected for the multilevel analysis were slice 2D area (cm^2) and surface porosity (%). The latter was calculated as the coefficient between total cell area and total crumb studied area in percentage.
Crumb colours were measured using the CIE-*L*a*b** uniform colour space by the means of a Minolta colorimeter (Chromameter CR-400/410, Konica Minolta, Tokyo, Japan) after standardisation with a white calibration plate (*L**= 96.9, *a**= -0.04, *b**= 1.84). Colour parameters indicate: *L** the lightness, *a** the hue on a green (-) to red (+) axis and *b** the hue on a blue (-) to yellow (+) axis (Matos & Rosell, 2013). Measurements were performed on three slices from each run.

2.2.5 Statistical analysis

Multilevel factorial analyses that encompass a correlation matrix, the regression analysis and the multivariate analysis of variance (MANOVA) were performed on the collected parameters using Statgraphics Centurion XVI (Statpoint Technologies, Inc. Virginia, USA).

3. Results and Discussion

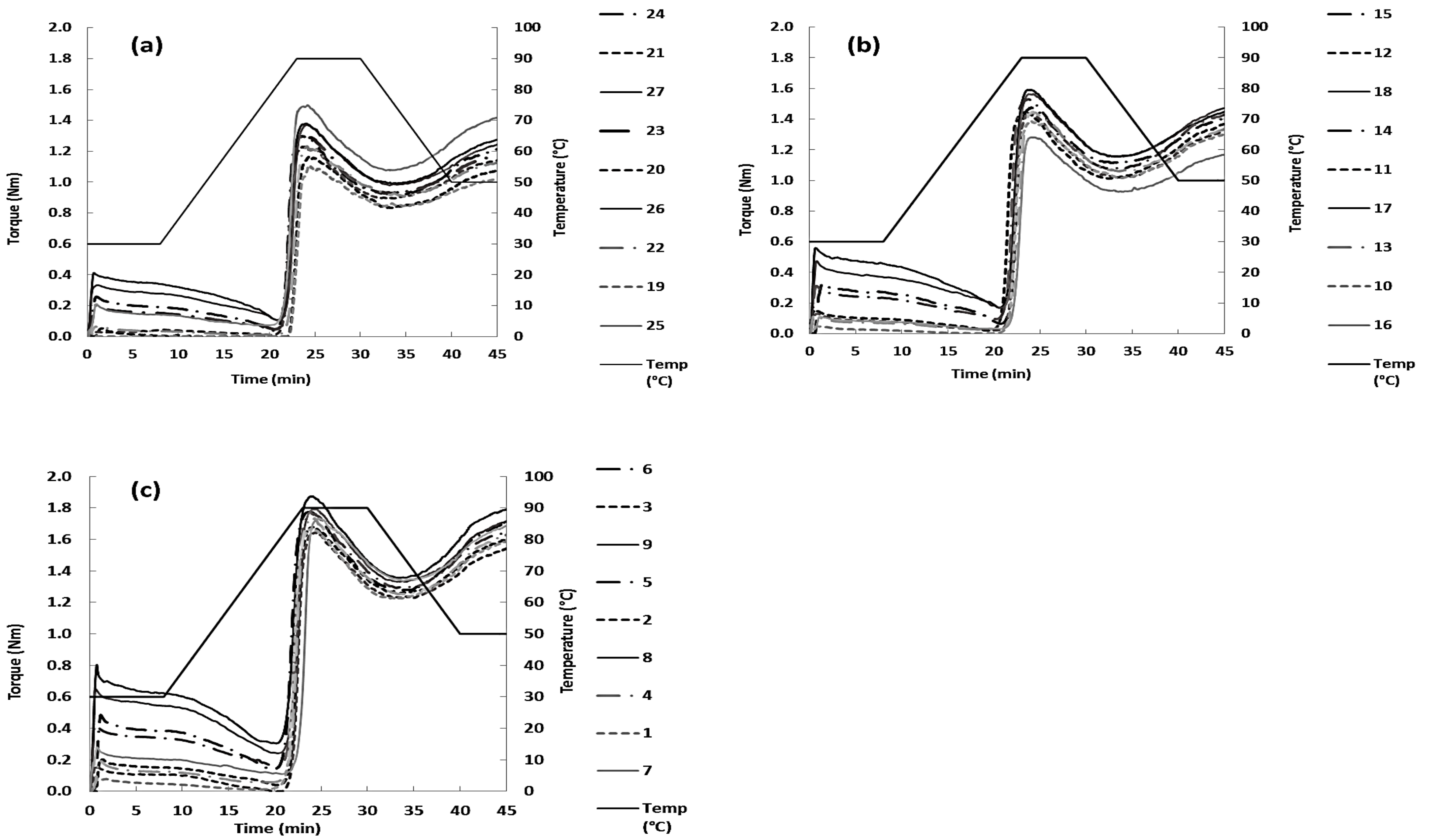
To identify the importance of hydrocolloid viscosity, a selection of commercial HPMC types with the same backbone structure and dissimilar chemical modifications were used. Levels of hydrocolloid and hydration were selected based on previous reported results (Matos & Rosell, 2013).

3.1 Effect of both hydrocolloid viscosity and level and hydration on the rheology of GF batters

Primary clear effect of hydration level on GF batters is shown in plots of the torque force recorded from the Mixolab® (Figure 1). Variations induced by the hydration level allowed us discriminating the runs into three different patterns that characterised the viscoelastic behaviour of batters through mixing, cooking and cooling phases, although the greatest differences were observed during mixing stage. Specifically, during mixing the water added to rice flour mixtures forms hydrophilic bonds with HPMC conferring some consistency to the liquid batters. It seems that the extent to which these bonds were formed depended primarily on HPMC viscosity. Therefore, mixing phase exhibited the highest inter-variability between curves.

Interestingly, according to the plots, differences in HPMC viscosity may be bridged with the increment of HPMC level. Throughout the successive stages, inter-variability among curves seemed to decrease, but observed relationship between HPMC viscosity and HPMC level was maintained. Mixolab® plots were analysed and parameters characterizing GF batters during mixing (C1 and C2), cooking (C3, C4 and breakdown) and cooling (C5 and set-back) were used for modelling their rheological behaviour.

Fig. 1 – Mixolab curves representing the rheological behaviour of the 27 GF batters. Plots are stratified and displayed according to the three levels of hydration used: (a) 110 %; (b) 100 %; (c) 90 %.



Analytical data (dependent factors) were fitted to multiple regression equations using HPMC viscosity, HPMC level and hydration level as independent factors. Table 2 shows the good fitting of the model for all the dependent factors and the significant regression coefficients of this dependence. Hydrocolloid acts as thickening and binding agent when included in rice-based GF mixtures, creating viscous systems and influencing the diverse phases of the GF bread making process (Houben et al., 2012; Sivaramakrishnan et al., 2004). In fact, viscosity of the hydrocolloid had a positive and linear effect on mixing (C1, C2) and cooling (C5, setback) stages, rising the batter consistency. In addition, viscosity had a positive and linear effect on total PA. Conversely, HPMC viscosity had a negative and linear effect during cooking (C3, C4, and breakdown). The quadratic effect of HPMC viscosity was negative for mixing and cooking parameters and total PA, whereas cooling phase and breakdown were not influenced. Concerning the level of HPMC, a significant linear and positive effect was observed for all the parameters of the mixing, cooking and cooling stages, whereas no quadratic effect was found. With this in mind, it seems possible to control the batter behaviour during mixing, cooking and cooling phases by recognising the role of the hydrocolloid viscosity along with the level of HPMC. When combined both factors, a synergistic effect on the parameters of mixing and cooking stages and on the total PA was observed, with the exception of breakdown. Beyond the interesting effect on the initial batter formation, results suggested the possibility to intervene effectively on some of the known issues of the cooking and cooling phases by considering always together the binomial hydrocolloid viscosity and level of hydrocolloid. Indeed through the HPMC viscosity it could be possible to control the GF batter consistency during cooking without increasing the rigidity of the GF system or interfere with the relationship among the swelled granules of starch, which cause their weakening and favour the breakdown (Mancebo et al., 2015). Moreover, it was possible to limit the starch breakdown also by delaying the water release and thus the swelling of the granules (Horstmann, Belz, Heitmann, Zannini, & Arendt, 2016), with possible positive consequences on the starch viscosity and crumb structure. According to the collected data from the parameters of cooling stage, HMPC ability to control the water retention and the consistency allowed managing some important quality characteristics of GF bread such as starch retrogradation (Crockett et al., 2011), moisture migration from crumb to crust and bread staling (Bárcenas & Rosell, 2005; Capriles & Arêas, 2014; Guarda, Rosell, Benedito, & Galotto, 2004). Even in the cooling phase, the observed HPMC viscosity effects were similar to those observed for HPMC level.

If looking for consistency, the increase of hydration has a negative influence, observing a significant negative correlation with all the rheology parameters, with the exception of breakdown. Therefore, results confirmed the critical role of an adequate hydration level in determining the strength of the batter three-dimensional structure. Significant positive quadratic effect of hydration was observed only in the case of minimum consistency during heating (C4). Significant antagonistic effects were observed between hydration and hydrocolloid viscosity and with the level of hydrocolloid on dough consistency during mixing (C1, C2) and the total working energy needed for the whole process. These results are in line with the evidence of the interaction between water molecules and HPMC that can contribute to build a three-dimensional structure with defined water-holding properties (Houben et al., 2012). To this regard, previous studies underlined that rising the level of hydration may weaken the three-dimensional structure formed by hydrocolloids with important consequences on batter and bread rheology (Mancebo et al., 2015). Present results confirm that assessment, but only during mixing because no significant effects were detected when subjected the system to heating and cooling.

Table 2 - Significant coefficients (95% confidence interval) of the independent factors of the stepwise regression fitting model for the rheological behaviour of the GF batters.

Factor	Mixolab® parameters							
	C1 (Nm)	C2 (Nm)	C3 (Nm)	C4 (Nm)	C5 (Nm)	Breakdown (Nm)	Setback (Nm)	Total PA (kWh)
Constant	0.6470	0.5296	5.4211	4.9963	5.6922	0.4248	0.6959	183.24
HPMC viscosity	0.2861***	0.1361***	-0.0250***	-0.0027***	0.0016***	-0.0222**	0.0044**	18.52***
HPMC level	0.6228***	0.3439***	0.0350***	0.0261***	0.1038***	0.0088**	0.0778*	36.29***
Hydration	-0.0126***	-0.0133***	-0.0562***	-0.0612***	-0.0646***	0.0051***	-0.0033***	-2.31***
HPMC viscosity^2	-0.0594***	-0.0256***	-0.0283**	-0.0138*	-	-	-	-5.09***
HPMC level^2	-	-	-	-	-	-	-	-
Hydration^2	-	-	-	0.0002**	-	-	-	-
HPMC viscosity *HPMC level	0.0750***	0.0267***	0.0175**	0.0125**	-	-	-	5.61***
HPMC viscosity *Hydration	-0.0032***	-0.0016***	-	-	-	-	-	-0.21**
HPMC level *Hydration	-0.0043***	-0.0031***	-	-	-	-	-	-0.23***
Adj. R-squared[a]	0.9797	0.9454	0.9839	0.9853	0.9800	0.6931	0.7074	0.9757

*P<0.05; **P<0.01; ***P<0.001. [a]adjusted square coefficient of the fitting model. Independent factors were HPMC viscosity, HPMC level and Hydration level.

3.2 Effect of hydrocolloid and hydration on the textural features of the bread

Texture, crumb porosity and moisture were selected as appropriate indicators of the bread quality. Experimental data collected from the twenty-seven breads formulated according to the multilevel factorial model were fitted to multiple regression equation using HPMC viscosity, HPMC level and hydration level as independent factors. Significant dependences are reported in Table 3. The factorial model expressed lack of fit (low levels of R-squared values) for surface porosity and moisture, and moderate significance for slice 2D area, adhesiveness and springiness. The effect of HPMC level on the slice 2D area indicated that higher bread volume was obtained by increasing hydrocolloid amount, which also originated higher batter consistency (C1). Conversely, a good fitting was observed for crumb hardness, cohesiveness and resilience. Taking into account the strong fittings, HPMC viscosity had positive linear effect on cohesiveness and resilience, whereas a negative effect on crumb hardness was observed; and positive quadratic effect on resilience. Similarly, to what was observed for rheology parameters, the impact of HPMC level closely followed the pattern of HPMC viscosity. Accordingly, the HPMC level may improve those parameters that typically represent a problematic issue in GF bread such as high crumb hardness, low cohesiveness and resilience. Interestingly, HPMC viscosity in combination with HPMC level had a positive effect on hardness and resilience, implying that when high viscosity HPMC is used at high level, harder crumbs with great ability to recover after compression will be obtained. Some previous studies observed that the presence of HPMC in GF batters is followed by several improvements in crumb textural properties of GF bread, like maintaining the homogeneity of the system, enhancing the interfacial activity during proofing and retaining gas during baking (Crockett et al., 2011; Hager & Arendt, 2013; Lazaridou et al., 2007). Present results highlighted that the desirable manipulation of parameters such as hardness, cohesiveness and resilience, might consider also the range of viscosities offered by HPMC.

Table 3 – Significant coefficients (95% confidence interval) of the independent factors of the stepwise regression fitting model for the GF bread quality characteristics.

Factor	**Instrumental quality parameters**							
	Slice 2D Area (cm^2)	**Surface porosity (%)**	**Hardness (g)**	**Cohesiveness**	**Adhesiveness**	**Resilience**	**Springiness**	**Moisture (%)**
Constant	15.59	35.72	-5198	1.300	-103.296	0.946	0.281	349.12
HPMC viscosity	-	-	-325***	0.053***	-	0.046***	-	-
HPMC level	3.09***	-	-304***	0.048*	-	0.003***	-	-
Hydration	-	-	145***	-0.010***	-6***	-0.009***	0.009***	-6.34**
HPMC viscosity^2	-	-	-	-	-124***	0.014***	-0.024***	-
HPMC level^2	-0.83**	-	144**	-	-67**	-	-	-
Hydration^2	-	-	-	-	-	-	-	-
HPMC viscosity*HPMC level	-	-	108**	-	-	0.004**	0.011**	-
HPMC viscosity*Hydration	-	-	-	-	5**	-0.001**	-	-
HPMC level*Hydration	-	-	-	-	-	-0.001*	-	-
Adj. R-squared[a]	0.6159	0.0000	0.7124	0.8644	0.6847	0.9402	0.4570	0.1295

*P<0.05; **P<0.01; ***P<0.001. [a]adjusted square coefficient of the fitting model. Independent factors were HPMC Viscosity. HPMC level and Hydration level.

Regarding hydration level (Table 3), it negatively influenced important GF bread quality parameters such as hardness, cohesiveness and resilience. In combination with HPMC viscosity or HPMC level, hydration level had an antagonistic effect on resilience. The effect of hydration on instrumental quality parameters of GF bread may find explanation in the above-discussed role of water in weakening the resulting three-dimensional structure of GF batter containing HPMC, which can dramatically affect the proofing and the crumb texture after baking, as revealed the analysis. Present results on antagonistic effect of hydration level and HPMC agreed to what observed by Mancebo et al. (2015), i.e. as far it concerns the inverse relation between hydration level and crumb hardness.

3.3 Relationship between rheological parameters of the formulated GF batters and instrumental parameters of the resulted bread.

Correlations found between rheological parameters of the GF batters and bread quality parameters are reported in Table 4. Only significant moderate ($0.3 \leq r \leq 0.7$) and strong ($r > 0.7$) correlation coefficients are shown. Slice 2D area showed positive and moderate correlations with C1, C2, C3, breakdown and total PA parameters. No significant correlation was found for surface porosity, which was expected since all the doughs were subjected to the same mixing speed and thus aeration, but results confirmed that no rare behaviour was induced by the independent factors tested. It is worthy to note that slice 2D area is a quality indicator of the magnitude of the opened crumb texture, which follows a good expansion of the batter during leavening (Hager & Arendt, 2013).

No significant correlation existed among crumb hardness and rheology parameters, although previous studies found strong correlation between crumb hardness and Mixolab® parameters in GF systems with diverse type of hydrocolloids (Matos & Rosell, 2013). Discrepancies might be ascribed to the fact that in the present study different batter consistencies were obtained with a range of HPMC that provided diverse viscosities, whereas in the reported study batter consistencies resulted from the presence of diverse types of hydrocolloids (xanthan gum, HPMC, pectin).

Cohesiveness showed moderate and positive correlation with all the collected Mixolab® parameters. Lack in cohesiveness and crumbly texture usually characterise the GF bread (Mancebo et al., 2015). Resilience, instead, displayed moderate and positive correlation with parameters related to cooking and cooling phases of the Mixolab®, whereas adhesiveness exhibited negative and moderate correlation with the same parameters. Negative and moderate correlation was found between springiness and C3, C4 and C5 parameters from Mixolab®. Low resilience and springiness negatively affect the elasticity and perception of freshness of the GF breads (Cornejo & Rosell, 2014). Therefore, the possibility to control the texture parameters such as cohesiveness and resilience by defining the adequate batter consistency could be convenient for GF bread making.

Table. 4 – Coefficients of significant[a] correlation between Mixolab® parameters and instrumental quality parameters.

	Instrumental quality parameters										
Mixolab® parameters	**Slice 2D Area (cm^2)**	**Surface porosity (%)**	**Hardness (g)**	**Cohesiveness**	**Adhesiveness**	**Resilience**	**Springiness**	**Moisture %**	***L****	***a****	***b****
C1 (Nm)	0.6233***			0.6997***				-0.3977*	0.6452***	0.5878**	0.4215*
C2 (Nm)	0.5911**			0.6054**				-0.4067*	0.5884**	0.5653**	0.4368*
C3 (Nm)	0.3952**			0.6068**	-0.5828**	0.5515**	-0.4411*	-0.4308*		0.6565***	0.7260***
C4 (Nm)				0.5697**	-0.6193***	0.5626**	-0.5009**	-0.4357*		0.6715***	0.7565***
C5 (Nm)				0.6084**	-0.5944**	0.5773**	-0.4828*	-0.4283*		0.6637***	0.7298***
Breakdown (Nm)	0.4552*			0.6208**	-0.4014*	0.4431*				0.5225**	0.5405**
Setback (Nm)				0.6362***	-0.4559*	0.5458**				0.5621**	0.5702**
Total PA (kWh/kg)	0.5804**			0.7170***				-0.4204*	0.5405**	0.6486***	0.5656**

*

P<0.05; ***P*<0.01; ****P*<0.001, indicate the significant correlation.

As previously highlighted by Matos & Rosell (2013), moisture was negatively correlated with C1 and gelling (C4-C5) parameters from Mixolab®. In addition, present study found moderate and negative correlation of moisture with C2 and C3, but not with breakdown and setback.

Crumb colour such as $L*$, $a*$ and $b*$ parameters were portrayed as important factors to be considered in GF bread quality characterisation (Cornejo & Rosell, 2014), because they can influence the consumer's acceptability of the bread. Present recipes contained white rice flour, without further ingredients that could alter the colour of the slice. In particular, $L*$ parameter was positively correlated with C1, C2 and total PA, whereas $a*$ and $b*$ present both positively moderate and positively strong correlation with all the Mixolab® parameters. These results indicate that the brightness of the crumb was not so much influenced.

3.4 Optimization

After studying the synergy among independent factors used (level and viscosity of HPMC and hydration) and the correlation between rheological and technological parameters of GF batter and bread, results were scored to obtain a desirability index representing a GF breads with optimum quality. For this purpose, representative rheological and textural parameters were chosen and valued as quality indicators according to Matos and Rosell, (2013). In particular, the minimization of C4 and C5 was chosen to represent the rheological parameters of the GF batter, whereas minimization of crumb hardness, maximization of crumb cohesiveness, springiness and 2D slice area of bread were selected for the textural parameters of GF bread.

Optimization analysis generated a desirability index of 0.726, referred to a GF system with optimum values for the independent factors studied, specifically HPMC viscosity 15000 cP (HPMC K15), HPMC level of 2.2% and optimum hydration of 110 %.

4. Conclusion

It is widely accepted that the inclusion of HPMC into GF breads formulation allows improving batter consistency and several qualitative characteristics of the product. Despite HPMC level and hydration level have always been the most used parameters to optimize the consistency of the GF batters, results brought about that other features of the HPMC can affect the batter rheology and quality of the GF breads. Hydrocolloid viscosity and level, besides batter hydration should be altogether defined when designing lean rice-based formulation. In particular, it is possible to accurately define a value for the hydration level and reduce the amount of HPMC to be used during. Therefore, a more accurate selection of the type of HPMC to be used in GF bread formulations is advisable.

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Chapter 4

Gluten-free pasta incorporating Chia (*Salvia hispanica L.*) as thickening agent. An approach to naturally improve the nutritional profile and the in-vitro carbohydrate digestibility.

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Abstract

A gluten-free pasta was prepared adding Chia at rice flour for testing the thickening and nutritional properties of this specie. Chemical analysis showed Chia as source of protein content (19.52% and 15.81%, seeds and mucilage respectively), insoluble/soluble dietary fiber ratio (4.3 and 1.79 seeds and mucilage respectively), fat and ash content. The total phenolic acids content ranged from 734.5µg/g and 923.9 µg/g for seeds and mucilage respectively.

Chia was a good thickening agent and, improved the nutritional profile of enriched samples in respect to commercial GF. After cooking TPAs increased in all samples, ranging from 5.3% in DW to 52.8% in CM5. Concerning the glycemic index lowering, the addition of chia seeds increased the slowly digestible starch fraction of rice, commonly known to have a high glycemic index. Results suggest that Chia should be added as thickening agent in the formulation of GF pasta for conferring healthier characteristics.

Running title: Nutritional characteristics of fresh gluten free pasta with chia

Keywords: Chia seed, Chia mucilage, Gluten-free fresh pasta, Phenolic Acid, Flavonoid.

Abbreviations: DW= durum wheat; CGF= commercial gluten free product ready for pasta making; CS5= 5% of milled Chia seeds replacement; CS10= 10% of milled Chia seeds replacement; CM5= 5% of Chia mucilage replacement ; CM10 = 10% of Chia mucilage replacement; PC= Protein Content; IDF= Insoluble dietary fiber; SDF= Soluble dietary fiber ;TPC= Total Phenolic Content; TPAs = Total phenolic acids; NRS= Not Resistant Starch; TS= Total Starch; RAG= Rapidly Available Glucose; SAG: Slowly Available Glucose; RDS= Rapidly Digestible Starch; SDS= Slowly Digestible Starch.

1. Introduction

In recent years gluten-free products have become more and more popular, fueling a growing market, as they not only cater to individuals with medical needs but also to the millions of consumers who believe that they are healthy goods (Pellegrini & Agostoni, 2015). This growing interest for these foods has led to improvements in rheological properties of dough for bread and pasta, which have been achieved through the addition of fats and other components to gluten-free flours. Because the content of proteins and antioxidants in traditional gluten-free flours is low, the use of nutrient- and dietary fiber-rich ingredients has been proposed to improve the nutritional quality of such foods and to reduce the glycemic response of gluten-free breads (Capriles, dos Santos & Arêas, 2015). Nevertheless, obtaining products as nutritious as wheat-based ones remains a challenge. For these reasons, research on new ingredients able to improve both technological and functional properties of gluten-free dough is therefore needed.

Chia *(Salvia hispanica* L) is an annual herbaceous species belonging to the *Lamiaceae* family and it was domesticated in Central America around 2600 B.C. In the past 25 years, it has been the object of a growing body of research (Reyes-Caudillo, Tecante & Valdivia-Lopez, 2008; Ayerza & Coates, 2009), which has established that Chia seeds are rich in poly-unsaturated fatty acids, antioxidants, vitamins, and minerals. The seeds contain over the 30% of oil and are among the richest natural source of the essential fatty acid α-linolenic [ALA; 18:3(n-3)]. Chia seeds provide a high-quality content of proteins (about 19-27%) with a good balance of essential amino acids, especially methionine and cysteine (Ayerza, 2013; Sandoval-Oliveros & Paredes-López, 2013). They are a good source of natural antioxidants, mainly phenolic acids (chlorogenic and caffeic acids) and flavanols (myricetin, quercetin, and kaempferol) (Amato et al., 2015).

Chia seeds are also rich in dietary fiber (Reyes-Caudillo et al., 2008), with a total content that ranges from 34% to higher than 50%, more than that of other grains such as flaxseeds (Reyes-Caudillo et al., 2008; Capitani, Spotorno, Nolasco, & Tomás, 2012). Dietary fiber is composed of polysaccharides and oligosaccharides associated with plant cell walls, which are water soluble (pectins, gums and others) and insoluble (lignin, cellulose, and others). These fibers have been recognized to exert several biological effects, such as delaying the glucose releasing from the food and the consequent decreasing of the post-prandial glycaemia. These parameters are strictly involved in lowering the incidence of diabetes and cardiovascular diseases (Schwingshackl & Hoffmann, 2013).

Like some other *Lamiaceae*, the dry fruits of Chia (seeds), when hydrated, exude a mucilage connected to the fruit outer cell layers (Muñoz, Cobos, Diaz, & Aguilera, 2012). It has been characterized as containing a high molecular weight tetra-saccharide (800-2000 kDa), tentatively identified as a polymer of (1→4)-β-D-xylopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-β-D-xylopyranosyl with 4-O-methyl-α-D-glucuronic acid ramifications in the O-2 position of β-D-xylopyranosyl in the main chain (Lin & Daniel, 1994). This mucilage is highly hygroscopic (Muñoz et al., 2012) and therefore it can increase the sense of satiety. Its rheological properties include thickening capacity (Capitani, Corzo-Rios, Chel-Guerrero, Betancur-Ancona & Nolasco 2015) and adhesion (Svec, Hruskova & Jurinova, 2015) and therefore it has been proposed as a component for biofilms for increasing the shelf-life of foods.

Upon addition of Chia to wheat bread, Iglesias-Puig and Haros (2013) measured a significant increase in the levels of proteins, lipids, ash and dietary fiber, and the inhibition of amylopectin retrogradation, which improves the shelf-life. Recently, the effect of Chia addition has been used for reducing the fat content in foods. Ferrari Felisberto et al. (2015) used Chia mucilage to replace some of the fat used in pound-cake without altering its technological characteristics. Silveira Coelho and Salas-Mellado (2015) used Chia seeds or flour to reduce the use of hydrogenated fats in breads, and thereby increased the content of fiber and ω-3 fatty acid and the ratio of polyunsaturated and saturated fats. Seeds and mucilage of Chia therefore may have the potential to increase the functionality of food, however also to interfere with its technological characteristics.

Since to enhance the nutritional properties of GF products (pasta and bread) as well as to improve their sensory properties remain an open challenge (Capriles et al. 2015), in this work we evaluated the potential of Chia as an innovative thickening agent for functional gluten-free fresh pasta. With this aim, we tested the hypothesis that the adding of chia seeds or extracted mucilage to rice (a gluten-free flour) improves the nutritional profile and the bioactive compound content in comparison with commercially available gluten-free product ready for pasta making without negatively affecting the firmness. At last, we tested also the effect on *in vitro* carbohydrate digestibility of fresh pasta samples with Chia added.

2. Materials and methods

2.1 Field material

A *Salvia hispanica* L. long-day genotype (G8), obtained by Jamboonsri (2010) and Jamboonsri, Phillips, Geneve, Cahill, & Hildebrand, (2012) and available to the University of Basilicata through an agreement with the University of Kentucky, was grown at Atella – PZ - Italy (Lat. N 40°51'37,59'' Lon. E 15°38'49,43'') in 2014 on a Luvi-vertic Phaeozem (Iuss working group, 2006) loam soil with the following characteristics: sand (50µm – 2000 µm) 43.6%, silt (2 µm - 50 µm) 34.2%, clay (<2 µm) 22.1%; pH 6.8; N 1.9 g kg^{-1}; P_2O_5 50.3 g kg^{-1}; K_2O 1430 g kg^{-1}. The site has an average annual rainfall of 678 mm concentrated mainly during October-May. The crops were grown in triplicate plots, sown on 21/06/2014 and grown with non-limiting water supply.

2.2 Preparation of Chia seeds and mucilage

Chia mucilage was extracted according to Muñoz et al. (2012) with same modifications: eight samples of 100 g of whole seeds were placed in a beaker and distilled water was added in 1:40 proportion (w/v). The extraction temperature was maintained at 80°C and the mixture was stirred with a magnetic stirrer and hydrated for 2 h. Then the aqueous suspension was spread on a drying tray and exposed to temperature of 50°C for 10 h. The dried mucilage was separated from the seeds by rubbing over a 40-mesh screen. 200 g of chia seeds were dipped in N_2 liquid and ground with a Tecator 1093 Cyclotec (Foss Italia, Padova, Italy) sample mill (1mm sieves) to obtain the whole meal.

2.3 Pasta preparation

Six fresh pasta samples were tested. Four experimental pasta samples were prepared adding 5% and 10% of milled chia seeds (CS5 and CS10, respectively) and 5% and 10% of chia mucilage (CM5 and CM10, respectively) to rice flour purchased in a local market. The control gluten-free pasta was made from a commercial gluten-free product ready for pasta making (CGF) purchased in a local market. A pasta sample from durum wheat (DW) semolina of high commercial quality (protein content 14%, gluten content 11.2%, yellow index 30) was also prepared.

The CGF is an industrial product made for homemade preparations and consisted of the following ingredients: corn starch, skimmed milk powder, sugar, psyllium seed, guar gum and corn maltodextrins as thickening agents. The five gluten free meals were added with deionized water (73 mL/100 g of flour) to obtain a malleable dough and handmade into "tagliatella" shape by a homemade-pasta machine (Imperia, Torino, Italy). Semolina was added with a low amount of water (50 mL/100 g semolina) and processed in the same way of gluten free samples. The sizes of "tagliatella" were 1.5 mm thickness and 5 mm width.

2.4 Cooking quality of pasta samples

Cooking analysis of pasta, including cooking time and firmness, was performed following AACC approved method 66-50 (2000). Eight g (7 cm strands) of tagliatella were cooked in 250 mL of boiling distilled water. The cooking time (CT) was set at 2 minutes when the middle white core disappeared. The pasta firmness at the CT was measured by TA.XT plus texture analyzer, equipped with a 25-kg load cell (Stable Micro System, UK). Texture parameters were: pre test speed = 1.0 mm/s; test speed = 0.17 mm/s; post test speed= 10 mm/s. Five strands of cooked tagliatella were placed parallel and were sheared by the blade. Firmness of cooked pasta was determined as the force (g) required to shear the five strands. For each sample, measures of CT and firmness were performed in duplicate and triplicate, respectively.

2.5 Proximal analysis

For seeds and mucilage, the determination of insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) was based on AOAC Method 991.43 (AOAC, 1997) modified as in Reyes-Caudillo et al. (2008), by using dialysis instead of ethanolic precipitation and by using a sample weight of 0.1 g. The protein content (PC, %N x 5.70) was determined using the Kjeldahl method. The total fat and ash content were determined by AOAC (1997) Method 920.39 and 925.09 respectively.

All the analyses were performed on the meals as well as on the six corresponding pasta samples (uncooked and cooked) prepared. Samples of cooked pasta (20 g) were dried in an oven equipped with a vacuum pump to remove the humidity without increasing the temperature (30 °C; 760 mm Hg) and then ground with a Tecator 1093 Cyclotec (1 mm screen). All samples were then thermally sealed in bags at -20°C until the analyses.

Determination of insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) in DW, rice flour and pasta samples added with Chia was based on AOAC Method 991.43 (AOAC, 1997) as described in the Megazyme assay kit (Megazyme International Ireland). Determination of the total starch (TS) was carried out on all the raw and cooked pasta samples based on AOAC Method 2002.02 (McCleary & Monaghan, 2002) using the Megazyme K-RSTAR® kit (Megazyme International Ireland). The protein content (PC, %N x 5.70) was determined using the UNI 10274 method. The total fat content by acid hydrolysis in DW and rice flour was determined by AOAC Method 925.12 (AOAC, 1990). The ash and the moisture content were determined as percentage by means of the approved methods (AACC, 2000) 08-01 and 44-19, respectively.

2.6 Extraction and quantification of phenolic acids and flavonoids

Phenolic acids and flavonoids were extracted as reported previously by Fares, Platani, Baiano and Menga (2010). Samples were analysed on Agilent HPLC–System 1200 (Hewlett-Packard, Germany) equipped with a diode array detector DAD (Agilent, Waldbronn, Germany) according to the method proposed by Kim, Tsao, Yang and Cui, (2006). Phenolic separation was performed using a 250 x 4.6 mm, 5 µm particle size Zorbax SB-C18 column (Agilent, U.S.A.). The temperature of the column oven was set at 35 °C. A gradient elution was used with a mobile phase consisting of acetonitrile (solution A) and 1% acetic acid (solution B) as follows: 0 min %B= 100%; 20 min % B= 85%; 30 min % B= 70%; 50 min %B= 50%; 55 min %B= 0%; 60 min % B= 100%; isocratic elution of 100%

B, 60-65 min. The flow rate of the mobile phase was 1 mL/min, and the injection volume was 10 µL. The wavelengths used for the quantification of phenolics were 280, 320 and 360 nm and all quantifications were based on peak area of the following phenolics standards: *p*-hydroxybenzoic, chlorogenic, vanillic, caffeic, vanillin, siringic *p*-coumaric and ferulic acids, (+)-catechin, quercetin and resveratrol (Sigma-Aldrich Milano, Italy).

2.7 Total phenolic content assay

The extracts for the total phenolic content was carried out as follows: 1 g of sample was ground with 1.0 mm sieve, extracted with 8 mL of a mixture methanol/water/HCl (80:19:1 v/v/v), and ultrasonicated for 30 min. The mixture was centrifuged at 1000 *g* for 15 min. The Total Phenolic Content (TPC) was determined using the method proposed by Singleton and Rossi (1965). An aliquot of the extract (0.2 mL) was added to 1.5 mL of 10-fold diluted Folin-Ciocalteu reagent. The mixture was allowed to equilibrate for 5 min and then mixed with 1.5 mL sodium carbonate solution (60 g/L). After incubation at room temperature for 90 min, the absorbance of the mixture was measured at 725 nm. Acidified methanol was used as blank. The results were expressed as mg ferulic acid/g (FAE/g). A calibration line was built on the basis of solutions at known and increasing concentrations of ferulic acid (Sigma-Aldrich, Milano, Italy). Determinations were performed in triplicate for each extract and reported on dry matter basis.

2.8 In vitro carbohydrate digestibility

The *in vitro* carbohydrate digestibility was carried out in according to Englyst 's method (Englyst, Veenstra, & Hudson, 1996), determining the amount of glucose released after 20 min (G20) and after 120 min (G120) after incubation with digestive enzymes: pancreatin, amyloglucosidase (EC 3.2.1.3), invertase (EC 3.2.1.26), all enzymes were purchased from Sigma (St. Luis, MO, USA). Briefly, 2 g of cooked and minced pasta sample were solved in 10 mL of pepsin-guar gum solution (obtained mixing 5 g of pectin powder and 5 g of guar gum from Sigma in 1 L 0.05 M HCl) in capped tubes immersed in a shaker water bath for 30 min at 37 °C. Ten mL of 0.25 M sodium acetate buffer and 5 glass balls were added for each capped tube, in order to allow the disruption of the food particles. Five mL of an enzyme solution of pancreatin suspension (3.3 g in 22 mL of bi-distilled water), amyloglucosidase (3.6 mL of a solution 140 U/mL) and invertase solution (37.5 mg dissolved in 3.06 mL of bi-distilled water) were added to the samples. After 20 min (G20) and 120 min (G120) incubation, an aliquot of samples was drawn and immediately cooled on ice, in order to block the reaction. Samples were then centrifuged at 4 °C, 16,025 *g* for 5 min and supernatants containing glucose were read with a 2900D Biochemistry Analyzer glucose reader (YSI, Yellow Springs, OH, USA). Free Sugar Glucose (FSG) was determined by mixing 2 g of pasta sample with 20 mL of 0.1 M sodium acetate buffer (pH=5.2) and shacking vigorously in tapped tubes containing 5 glass balls each at 100 °C for 30 min. After cooling down the samples at 37 °C, 0.2 mL of 12.25 mg/mL invertase solution were added and tubes were allowed to shake for 30 min at 37 °C. One mL of sample was collected and, after centrifugation for 5 min at 16,025 *g*, supernatant was assayed for glucose content. The values of rapid digestible starch (RDS) and slowly digestible starch (SDS) were calculated as follows:

RDS: 0.9 x (G20-FSG)

SDS: 0.9 x (G120-G20)

2.9 Statistical analysis

Values are expressed as the mean ± standard deviation of three independent measurements. Significant differences among the measurements were assessed using the one-way analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) test ($p < 0.05$). Analyses were performed using the software STATISTICA 7.1 software (StatSoft Italia srl).

3. Results and Discussion

3.1 Nutritional composition and phenolic content in Chia and flours

Table 1 shows the nutritional composition and the phenolic profile of the Chia seeds and mucilage, DW, CGF and rice flour. The protein, insoluble and soluble dietary fiber, fat and ash contents of Chia seeds and mucilage were significantly higher than the other flours, showing the great relevance of this crop as source of nutrients. Our results on the content of protein, ash and lipids agreed with those found by Coelho and Salas-Mellado (2014), who analyzed chia seeds grown in Brazil, but were lower than those reported in the study of Marineli, Moraes, Lenquiste, Godoy, Eberlin and Maróstica (2014) around 25.3%. These differences can be attributable to the different variety of Chia as well as environmental factors. The lipid content of Chia seeds was similar to that reported by Amato et al. (2015) for different varieties of Chia grown in the same environment as this experiment. Chia represents a source rich in poly-unsaturated fatty acids, among which the essential fatty acid α-linolenic and linoleic acid (Marineli et al. 2014).

Table 1: Nutritional composition and phenolic profile of Chia seeds, Chia mucilage, durum wheat semolina, rice flour and gluten-free commercial flour.

Samples	PC (%)	IDF (%)	SDF (%)	Fat (%)	Ash (%)	TPC (mg FAE/g)
DW semolina	14.56±0.63 c	4.02±0.14 c	0.80±0.07 d	1.28±0.07 c	0.88±0.01 c	0.89±0.30 c
CGF	2.91±0.13 e	1.9±0.08 d	4.1±0.98 c	0.5±0.01 d	0.67±0.01 c	0.41±0.080 c
Rice flour	6.93±0.12 d	0.95±0.08 e	0.2±0.01 e	0.5±0.01 d	nd	0.65±0.006 c
Chia seeds	19.52±0.13 a	32.05±1.15 ab	7.41±4.05 b	31.56±0.92 a	4.5±0.02 b	3.21±0.190 a
Chia mucilage	15.81±0.04 b	32.9±0.89 a	18.36±3.06 a	2.27±0.16 b	10.1±0.02 a	1.36±0.006 c

Data are means of three determinations ± standard deviation and are expressed on d.m. Different letters in the same column indicate statistical differences by LSD test ($p < 0.05$).
Abbreviations: DW= durum wheat; CGF= commercial gluten free product ready for pasta making; nd= not detectable; PC= protein content; IDF= insoluble dietary fibre; SDF= soluble dietary fibre; TPC= total phenolic content.

The IDF values were in agreement with the results found by Reyes-Caudillo et al. (2008) in Sinaloa and Jalisco, two Chia varieties grown in Mexico. The high content of insoluble fiber is relevant from health standpoint as both lignin and insoluble hemicellulose are responsible of bile acids absorption, which, in turn, exert a lowering effect on LDL cholesterol level. Also, the SDF has potential benefits for health, as its intake is associated with prebiotic effects. The amount of SDF found in mucilage was 2.5-fold in respect to seeds, where we found an amount comparable with that determined by Reyes-Caudillo et al. (2008). As expected, the SDF content was very high, as it is the mucilaginous capsule formed when the seeds were soaked in water and is represented by non-starch branched polysaccharides, mainly constituted by xylose, glucose, and glucuronic acid (Munoz et al. 2012), which in turn are responsible of the gelling properties of chia.

A variation in values of chia mucilage proximal composition has been reported in the literature and Ferrari Felisberto et al. (2015) attribute differences in ash content to the different amounts of outer seed layer impurities found in chia gels extracted with different methods. The same applies to the content of fiber.

Regarding the antioxidant properties of the flours, we found very high and consistent levels of TPC in both Chia seeds and Chia mucilage. The TPC, which measures the total amount of polyphenols of each extract, showed that in seeds they were 2-fold higher (3.21 mg/g FAE) in respect to mucilage (1.36 mg/g FAE), while the amount detected in the other samples had a concentration less than 1 mg/g FAE (0.89 mg/g FAE, 0.41 mg/g FAE, 0.65 mg/g FAE for DW semolina, CGF and rice flour, respectively). The TPC measured were close to those of Martinez-Cruz and Paredes-Lopez (2014), who fond 1.65 mg GAE/g in Chia seed, but were higher than those found by Marineli et al. (2014), and Reyes-Caudillo et al. (2008), which turn around 0. 88 - 0.94 mg GAE/g.

Moreover, the high content of TPC can be explained also by the presence of several other antioxidants, especially tocopherols, abundant in the oil fraction of seeds (Amato et al., 2015), since no defatting treatment was applied to seeds.

The sum of phenolic acids and flavonoids amounted up to 923.9 µg/g and 734.5 µg/g for mucilage and seeds, respectively (Table 2). Our data agree with Reyes-Caudillo et al. (2008) who reported a range variable of phenolic compounds in Chia seeds ranging from 551 to 881 µg/g.

In this regard, in Table 2 are shown the phenolic acids profile and the flavonoids detected in the samples. In DW, a low amount of caffeic acid was found, while *p*-coumaric and, above all, ferulic acids were more abundant (8.36 µg/g and 96.44 µg/g, respectively). These quantities are consistent with our previous study on phenolic acid profile and antioxidant capacity of durum wheat pasta (Fares et al., 2010).

In the CGF only vanillin, *p*-coumaric and ferulic acids were found, while in rice flour only *p*-coumaric and ferulic acids were determined in low amount. These findings agree with the low antioxidant potentiality of both the CGF and rice flour. On the contrary, in Chia seeds and mucilage we found out a great variety and amount of phenolic acids, while for flavonoids, only catechin (7.06 and 8.4 µg/g in seeds and mucilage, respectively) was detected. Among the phenolic acids, caffeic acid was the most abundant and represented about 51.3% and 60% of the total amount in seeds and mucilage, respectively.

In Chia seeds, ferulic acid represented around 21.2%, while in mucilage about 16%; chlorogenic acid represented 10.3%, and 7.7% in seeds and mucilage, respectively, while *p*-hydroxybenzoic acid showed about the same concentration in both chia samples (5.8% and 6.2% respectively).

Despite Chia mucilage had a higher content of phenolic acids compared to Chia seeds (923.9 µg/g and 734.5 µg/g, respectively), the sum of chlorogenic and caffeic acids was similar and represents about 68% and 61% of the total. A higher content of phenolic compounds in the mucilage can be ascribed to the fact that it is a mostly fibrous fraction extruded from the outer layers of the chia fruit (Munoz et al., 2012), likely rich in phenols, whereas whole seed meal includes inner layers with a probably lower polyphenol content, so that the overall concentration is lower. Our study basically agrees with Reyes-Caudillo et al. (2008), who found that the most representative phenolic acids in Chia seeds were caffeic and chlorogenic acids. These two compounds may be responsible of the good oxidative stability of seeds than oil, as established by Amato et al. (2015) with the Oxitest on Chia seeds cultivated in the same environment.

Table 2: Phenolic acids and flavonoids in the analyzed flour samples.

	p-Hydroxybenzoic Acid (µg/g)	Catechin (µg/g)	Chlorogenic Acid (µg/g)	Vanillic Acid (µg/g)	Caffeic Acid (µg/g)	Syringic Acid (µg/g)	Vanillin (µg/g)	p-Coumaric Acid (µg/g)	Ferulic Acid (µg/g)	Phenolic compounds*
DW semolina					0.03±0.1			8.36±0.93	96.44±1.34	105.10±2.1 c
CGF							8.75±3.08	14.46±0.76	10.30±0.27	33.52±4.7 d
Rice flour								7.20±0.11	5.03±1.35	12.25±0.52 d
Chia seeds	45.55±1.57	7.06±1.28	75.70±11.91	21.67±3.00	376.75±82.32	5.61±0.35	34.36±2.14	15.27±1.78	155.56±5.72	734.54±90.01 b
Chia mucilage	54.32±1.31	8.40±1.02	70.84±0.02	31.02±7.14	557.24±59.26	7.51±3.30	14.30±0.99	30.97±0.87	149.25±8.24	923.86±75.56 a

Data are means of three determinations ± standard deviation and are expressed on d.m. * Data are the sum of phenolic acids and flavonoids. Abbreviations: DW= durum wheat; CGF= commercial gluten free product ready for pasta making. Different letters in the same column indicate statistical differences by LSD test ($p < 0.05$).

3.2 Nutritional properties and cooking quality of pasta

Our goal was to test the potential of Chia seeds and mucilage as a functional ingredient to produce a nutritious gluten-free fresh pasta with a high content of natural antioxidants, fiber, protein and with good rheological properties, including thickening capacity.

In Table 3 are shown the nutritional traits in both uncooked and cooked pasta samples. The total starch content of the pasta samples was determined and the durum wheat pasta content lied among the lowest in both raw and cooked samples (71.17% and 69.44%, respectively), whereas the gluten free commercial product among the highest in the both cooked and uncooked pasta samples (76.14% and 80.02%, respectively). These data are both in according to the USDA Food Composition Database (2016). In pasta samples added with Chia, data evidenced values ranging between 70% and 80. Pasta from DW semolina had the highest content of PC in raw as well as in cooked sample. Durum wheat is the best raw material for pasta making, and therefore this result was expected. The lowest PC was detected in pasta samples from CGF, with 2.94% and 2.90% in raw and cooked samples, respectively. In pasta samples with Chia, we found the PC about 3-fold higher than pasta from CGF. Moreover, the increase of PC paralleled the additions of seeds and mucilage, with a higher PC in 10% addition than in 5%. This trend was maintained after cooking. The fiber contents (IDF and SDF) of the six pasta treatments before and after cooking are shown also in Table 3. The lowest content of total fiber was detected in raw pasta from CGF (5.89%) followed by DW (7.22%), CS5 (8.36%), CS10 (8.59%) and CM5 (7.85%), while the highest content was in CM10 (10.18%).

After cooking, total dietary fiber content decreased in all pasta samples added with Chia (seeds and mucilage) except for CS10. This observed increase in CS10, was ascribable to IDF. Interestingly, raw pasta from CGF had the significantly highest content of SDF that halved after cooking (3.23% and 1.14%, respectively), while the IDF doubled (2.66% and 5.42%, respectively). The latter behavior could be related with the presence of corn starch in the formulation of CGF, which induced the formation of retrograded amylose after cooking measured as IDF. In DW pasta, the amount of IDF significantly increased after cooking (4.5% and 5.03% respectively), while the SDF decreased (from 2.72% to 2.30%). Generally, for all Chia enriched pasta treatments, a decreasing trend was observed in the IDF content after cooking, except for CS10. In the case of cooked pasta samples added with chia seeds (CS5 and CS10), the SDF decreased in respect to the corresponding raw samples, while in cooked pasta samples added with Chia mucilage (CM5 and CM10), the amount of SDF was unchanged.

Table 3: Nutritional properties and firmness of raw and cooked pasta samples.

Samples	TS (%)	PC (%)	IDF (%)	SDF (%)	Firmness (g)	MC (%)
Raw Pasta						
DW	71.17±0.41 cd	14.76±0.02 b	4.5±0.23 g	2.72±0.05 b		34.2±0.4
CGF	76.14±2.79 ac	2.94±0.11 g	2.66±0.38 h	3.23±0.122 a		37.3±0.34
CS5	78.65±0.66 a	7.53±0.02 ef	6.09±0.93 d	2.27±0.124 de		40.2±0.7
CS10	76.96±0.87 ab	8.18±0.04 c	6.55±0.06 c	2.04±0.0202 ef		40.0±0.14
CM5	72.45±0.22 bd	7.54±0.02 ef	5.36±0.07 ef	2.49±0.046 bd		41.8±0.11
CM10	70.47±3.32 d	8.09±0.13 cd	7.58±0.31 b	2.60±0.155 bc		41.9±0.04
Cooked Pasta						
DW	69.44±1.64 d	15.14±0.033 a	5.03±0.04 f	2.30±0.07 ce	1010.186±6.35 a	57.3±0.51
CGF	82.87±4.21 a	2.90±0 g	5.42±0.16 e	1.14±0.02 g	617.30±16.55 b	64.2±0.07
CS5	80.02±1.17 ab	7.80±0.09 de	5.23±0.07 ef	1.75±0.07 f	400.67±12.43 d	66.8±0.1
CS10	76.00±4.39 ac	8.38±0.01 c	5.38±0.01 a	1.88±0.09 f	526.57±10.11 c	65.0±0.6
CM5	74.89±2.86 ac	7.41±0.37 f	4.57±0.07 g	2.39±0.37 bd	406.68±10.19 d	71.6±0.67
CM10	70.89±4.27 bc	8.21±0.15 c	6.60±0.02 c	2.65±0.08 b	633.81±3.93 b	66.8±0.06

Data are means of three determinations ± standard deviation and are expressed on d.m. Different letters in the same column indicate statistical differences by LSD test ($p < 0.05$).
Abbreviations: DW= durum wheat; CGF= commercial gluten free product ready for pasta making; TS= Total Starch; PC= Protein Content; IDF= Insoluble dietary fiber; SDF=Soluble dietary fiber; MC= Moisture Content; CS5= 5% of milled chia seeds replacement; CS10= 10% of milled chia seeds replacement; CG5= 5% of chia mucilage replacement; CG10 = 10% of chia mucilage replacement.

In previous studies, an increase of total dietary fiber after cooking was measured in durum wheat pasta (Fares & Menga, 2012) because of the gelatinization and the formation of retrograded amylose, namely resistant starch, during cooking and subsequent cooling of pasta sample. Interestingly, this trend was not found in pasta samples with seeds and mucilage; most likely a more severe effect of leaching on fiber was exerted by boiling water in these samples in respect to durum wheat pasta, where the gluten network prevents this phenomenon. The moisture content (MC) of raw pasta showed homogeneous values among samples with chia added (seeds and mucilage), while in DW and CGF we found a lower content. After cooking both the CGF and pasta samples with Chia added showed similar moisture which differed from DW pasta where we found the lowest content. This behavior is due to the hydrophobic characteristics of gluten network that didn't allow a full hydration of starch. Regarding the cooking behavior, we tested the firmness, as it defines the most important evaluation index of pasta. Firmness is a measure of the force required for shearing five strands of cooked spaghetti, and in this case study, five strands of "tagliatella".

As expected, the best cooking behavior was that of DW pasta (1010.186 g), but outstanding, pasta from CGF as well as CM10 showed a comparable performance (617.3 g and 633.8 g, respectively).

The lowest values were found in CS5 and CM5 (400.67 g and 406.68 g, respectively). The lower value of firmness found in CS10 in respect to CM10 could be related to the presence of less fiber in this pasta sample. The firmness of durum wheat pasta is determined by gluten proteins that form a network through cross-linking mechanisms which increase the protein aggregation and the formation of additional inter-chain disulfide bonds (Wagner, Morel & Cuq 2011). In GF products the structure-building potential is carried by proteins, hydrocolloid and binding agents (Caprile et al., 2015). DF also possesses functional properties which improves the physical and sensory qualities of GF products and above all, SDF is highly desirable for health purpose as well as functional one (Caprile et al, 2015). It is known that the mucilage from chia represents a relevant source of gum with excellent rheological properties which includes thickening capacity (Capitani et al., 2015) and adhesion (Svec et al., 2015). Moreover, Chia also has a high protein content which are largely used in GF products to build network that mimics the gluten's properties (Caprile et al., 2015). Thus, both these components could have been involved in the mechanism controlling firmness of CM10. These results are encouraging and they validate the capacity of chia to be a valuable structuring agent for gluten free flours. Furthermore, we also showed the improvement of the nutritional profile of enriched pasta. Up to now many attempts have been focused on bread (Capriles et al., 2015) and our work represents the first research on the application of chia seeds and mucilage as alternative ingredients to make healthier the gluten free pasta.

The phenolic acids profile of the raw and cooked pasta samples is summarized in Table 4. The highest contents of TPAs in raw samples were detected in pasta from CS10 and DW, which did not significantly differ one from the other, followed by CM10 pasta sample; the lowest content was found in pasta from commercial gluten-free flour (10.30 µg/g). These differences are ascribable to the qualitative and quantitative compositions of phenolic acids. In particular, these results may be attributable to the absence of caffeic and chlorogenic acids in DW and CGF, as compared to Chia pasta samples with the highest enrichment of seeds and mucilage (CS10 and CM10). The maximum yield of ferulic acid instead was found in DW pasta, but it was also abundant in pasta samples added with chia seeds and mucilage. Raw pasta from CGF showed the lowest content of all phenolic acids. Several researches have ascertained the prevalence of ferulic acid in durum wheat (Mateo Anson, Van Der Berg, Havenaar, Bast, & Haenen, 2008; Fares et al., 2010), which in turn is responsible of the antioxidant activity of pasta (Fares et al., 2010; Fares et al., 2012). On the other hand, Chia seeds represent a valuable source of caffeic and chlorogenic acids (Marineli et al., 2014). After cooking, the amount of TPAs increased in all pasta samples, showing an increase of 5.3% (DW), 14.8% (CGF), 25.5% (CS5), 13.7% (CS10), 52.8% (CM5) and 34.7% (CM10). The highest amount was detected in CS10 and CM10 which confirmed their relevant nutritional profile before as well as after the cooking process. The observed variation of the TPAs in all cooked pasta samples is mainly ascribable to the increased extraction of bound phenolic acids after boiling (Fares et al., 2010).

Table 4: Phenolic acids profile of raw and cooked pasta samples.

	p-Hydroxybenzoic Acid (µg/g)	Chlorogenic Acid (µg/g)	Caffeic Acid (µg/g)	Vanillin (µg/g)	p-Coumaric Acid (µg/g)	Ferulic Acid (µg/g)	TPAs (µg/g)
Raw Pasta							
DW	0.66±0.02				3.66±0.34	144.77±2.46	149.08 bd
CGF	0.56±0.32		1.04±0.08	0.38±0.06	2.33±0.24	5.99±0.30	10.30 g
CS5	1.95±1.22	2.25±0.28	6.48±3.51	1.88±1.08	5.71±1.86	79.88±1.46	98.40 f
CS10	3.14±0.47	4.56±0.02	36.48±1.61	3.42±0.14	6.99±0.09	109.45±0.7	164.3 b
CM5	3.43±0.15	0.28±0.01	13.12±0.83	0.51±0.08	5.51±0.010	71.28±0.23	94.50 f
CM10	3.95±0.22	1.38±0.16	43.29±0.74	0.96±0.03	6.35±0.07	81.70±6.77	138.12 de
Cooked Pasta							
DW	0.45±0.02				3.77±0.16	152.77±6.89	156.99 bc
CGF	0.39±0.32		1.56±0.37	1.67±0.15	3.07±0.45	5.15±0.53	11.83 g
CS5	2.24±1.47	2.50±0.29	15.99±7.62	2.83±0.05	6.27±2.18	93.32±11.57	123.53 e
CS10	3.66±0.21	4.98±0.30	41.82±0.21	4.05±0.09	7.98±0.11	123.88±0.02	186.80 a
CM5	5.45±0.39	0.92±0.21	20.40±0.45	0.35±0.02	7.90±0.07	108.96±0.02	144.40 cd
CM10	2.35±0.05	4.63±0.01	40.21±2.29	5.02±0.02	7.87±0.26	125.47±0.02	186.0 a

Data are means of three de terminations ± standard deviation and are expressed on d.m. Different letter s in the same column indicate statistical differences by LS D test ($p < 0.05$).
Abbreviations: DW= durum wheat; GF= commercial gluten free product ready for pasta making; TPAs = Total phenolic acids; CS5= 5% of milled chia seeds replacement; CS10= 10% of milled chia seeds replacement; CM5= 5% of chia mucilage replacement; CM10 = 10% of chia mucilage replacement

3.3 In vitro carbohydrate digestibility

The histogram in Figure 1 is relative to the *in vitro* carbohydrate digestion of pasta samples and shows the percentages of starch released after 20 min (RDS) and 120 min (SDS). Durum wheat and commercial gluten free pasta samples showed similar results, with an estimated equal content of rapidly and slowly digestible starch. This result is in accord with Englyst, Hudson and Englyst, (2000), only for the durum wheat pasta, while, for gluten free commercial pasta, there are no specific references in literature.

Concerning pasta samples added with Chia, results evidenced a significant higher value of rapid digestible starch compared to the slow digestible one for all the tested samples. This result may be explained by considering that rice, the flour to which chia was added, has small starch granules and, once damaged, they increase water absorption, becoming more susceptible to enzyme hydrolysis and glucose release (Hager, Wolter, Jacob, Zannini & Arendt, 2012).

In relation to Chia addition to rice flour, results evidenced that the total amount of CM5 starch is rapidly digestible (25.75%), while CM10 is characterized by 3.12% of SDS and 18.43% RDS; similarly, the increasing concentration of Chia seeds in pasta samples increases the SDS fraction: CS5 accounted for 3.46% SDS and 21.11% RDS, while CS10 had 6.02% SDS and 16.32% RDS. Intriguingly, CS pasta samples, in both the added concentrations, had a higher value of slow digestible starch compared to CM pasta, although the firmness is slightly lower for this latter kind of samples (Table 3). Chia seeds are extremely rich in fats, with more than 30% of the dry weight by oil (Amato et al. 2015), which are not transferred to the mucilage because of the water boiling process. Guraya, Kadan and Champagne (1997) demonstrated that amylopectin is able to complex with long chain mono- and polyunsaturated fatty acids and, after the boiling process, evidenced an increase of viscosity and a reduction of the digestibility. This effect may be due to a barrier effect made by lipids which counteracts the amylases degradation of the starch, causing a delay in the starch digestibility.

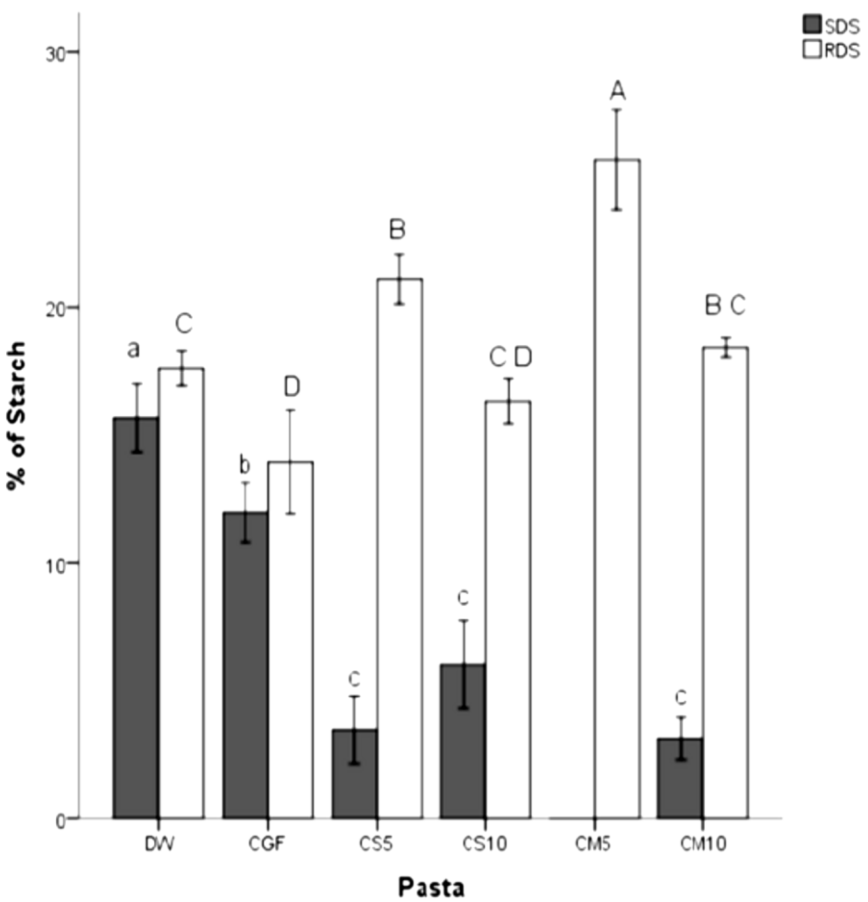

Fig. 1. In vitro carbohydrate digestion of pasta samples.

Data are means of three determinations ± standard deviation. Different letters indicate statistical differences by LSD test ($p < 0.05$) between SDS (not capitalized letters) or RDS (capitalized letters) values.

Abbreviations: DW = durum wheat; GF = gluten-free commercial product ready for pasta making; CS5 = 5% of milled Chia seeds replacement; CS10 = 10% of milled Chia seeds replacement; CG5 = 5% of Chia mucilage replacement; CG10 = 10% of Chia mucilage replacement.

4. Conclusions

Wheat-based products are staple food items in the diet of most people all over the world and obtaining gluten-free pasta with similar characteristics to wheat-based products represents a difficult task for industry. Gluten-free products are usually very rich in starch and lack in proteins. In a recent review (Capriles & Arêas, 2016) it was stressed the urgency to reduce the glycemic index of gluten-free foods, through the introduction of raw materials rich in fiber and protein. Our research pursues those expectations and proposes to overcome this problem by adding chia seeds and mucilage to gluten-free flour, like rice, for gluten-free fresh pasta. The research has demonstrated that Chia can represent a valid substitute of hydrocolloids (which mimic the gluten network) in the formulation of gluten-free pasta. Our results are encouraging, as have shown that it is possible to obtain a gluten-free pasta with cooking characteristics equivalent to those achievable from a commercial product as confirmed by the values of firmness on cooked samples. Moreover, with both mucilage and seeds 10% of Chia we have obtained pasta samples more nutritious and healthy in respect to pasta from CGF as confirmed by the high content of protein, dietary fiber and phenolic acids.

Concerning the key point of the glycemic index lowering, the addition of the Chia seeds revealed to increase the slowly digestible starch fraction of the rice, commonly known to be a high glycemic index cereal (Englyst et al, 1996), and in turn the nutritional properties of one of the most commonly flour used for the gluten-free products preparation. However, further *in vitro* and *in vivo* studies are required to test the expected reduction on glycemic index and glycemic load of these preparations.

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Chapter 5

Are the dietary habits of treated individuals with celiac disease adherent to a Mediterranean diet?

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Abstract

Background and Aims: The only treatment for celiac disease (CD) is strict, lifelong adherence to a gluten-free (GF) diet. To date, there are contrasting data concerning the nutritional adequacy of GF products and diets. There have been no studies that have assessed the adherence of individuals with CD to a Mediterranean diet (MD), a protective dietary regimen against major non-communicable diseases (NCDs). Therefore, we examined the adherence to an MD of a group of Italian individuals with CD and compared it with that of a healthy control group.

Methods and Results: In a cross-sectional study, a sample of individuals with CD on a stable diet for more than two years and a group of healthy subjects were included. The dietary habits of all participants were recorded using a validated food frequency questionnaire, and the adherence to an MD was determined using the Italian Mediterranean Index. Typical Mediterranean food consumption was not significantly different between individuals with CD and the healthy participants, except for fruits (P = 0.017). However, individuals with CD consumed significantly higher amounts of potatoes (P = 0.003) and red and processed meats (P = 0.005) than healthy participants. The resulting mean Italian Mediterranean Index was significantly higher in healthy participants than in individuals with CD (P <0.001).

Conclusion: The results raise questions concerning the food choices of individuals with CD suggesting the need of encouraging them to make better food choices more in line with an MD, which would improve their nutritional status and better protect them from NCDs at long term.

Protocol registration: ClinicalTrials.gov (ID NCT01975155).

Keywords: individuals with celiac disease; gluten-free diet; Mediterranean diet; Italian Mediterranean Index; nutritional quality.

Abbreviations used: NCDs, Non-Communicable Diseases; MD, Mediterranean Diet; CD, Celiac Disease; GF, Gluten-Free; FFQ, Food Frequency Questionnaire; EPIC, European Prospective Investigation into Cancer and Nutrition.

1. Introduction

Diet is a major factor in the promotion and maintenance of health throughout the entire life course and in the prevention of non-communicable diseases (NCDs), such as diabetes, cancer, obesity, and cardiovascular and respiratory diseases. Different approaches can be used to investigate the relationship between dietary habits and NCDs risk. Among them, dietary pattern approaches are now frequently used in nutritional studies, since people do not eat single foods or nutrients [1]. Indeed, over the past years, adherence to the whole diet rather than single component analysis has been devoted to exploring such a relationship. Accordingly, the a priori dietary pattern approach, also named the "investigator-defined dietary pattern", has been applied to investigate the effect of the Mediterranean diet (MD) on several NCDs [2]. This traditional dietary pattern, encountered in the early 1960s in southern Europe, has been recently stated as an intangible heritage of humanity by United Nations Educational, Scientific and Cultural Organization (unesco.org). It is a frugal diet characterized by high intake of cereals, fruits, nuts, vegetables, legumes, and olive oil; moderate intake of fish and poultry; low intake of dairy products, meat, processed meats, and sweets; and moderate consumption of wine with meals [3,4]. The epidemiological evidence has shown that adherence to an MD can significantly decrease the risk of overall mortality and the incidence of NCDs [3–6]. Consequently, an MD or a Mediterranean-like dietary pattern has acquired public health and clinical relevance for primary and secondary prevention of major NCDs in the overall population [4].

Celiac disease (CD) is a chronic systemic autoimmune disease triggered by the ingestion of gluten. In individuals with CD, the ingestion of foods containing gluten results in immunologically mediated inflammatory damage to the small-intestinal mucosa. Nowadays, the only treatment for CD is strict, lifelong adherence to a gluten-free (GF) diet that promotes intestinal healing and symptom relief and usually prevents complications of CD [7]. A GF diet is characterized by a combination of naturally occurring GF foods with GF substitutes for cereal-based foods [8].

Since the only restriction is the exclusion of gluten, the GF diet composition is based on personal food choices. Currently, there are contrasting data about the nutritional adequacy of a GF diet in terms of nutrients and food categories [9–12]. Moreover, the long-term metabolic consequences of GF diet on treated patients are largely unknown.

However, to the best of our knowledge, no studies have adopted the a priori dietary pattern approach to assess the MD adherence of individuals with CD. Therefore, we examined the adherence to an MD, as determined by the Italian Mediterranean Index [5], of a group of Italian individuals with CD and compared it with that of a healthy control group.

2. Material and Methods

2.1 Participants and Study Design.

Patients with CD were recruited from among the subjects referred to the Center of Prevention and Diagnosis of Celiac Disease at the IRCCS Cà Granda Foundation, Policlinico Hospital, Milan, as previously described [12]. The exclusion criteria were: (1) diagnosis of CD less than two years prior, (2) age under 18 or over 70, (3) metabolic or chronic disease (diabetes mellitus, Crohn's disease, cardiovascular and neurovascular diseases, cancer, neurodegenerative diseases, rheumatoid arthritis), (4) pregnancy or lactation, or (5) vegetarianism. Non-celiac persons (healthy participants) were recruited among students, researchers, and professors of the Universities of Parma and Milan.

Exclusion criteria for the healthy participants were the same except for the diagnosis of CD. All individuals were recruited between October 2012 and August 2014, and the data were collected during the same period. Of the 220 healthy participants who expressed interest in the study, 190 were eligible for the study and invited to participate. The enrollment process of individuals with CD has been previously described [12]. Two hundred individuals with histologically confirmed CD, all adhering to a strict GF diet as confirmed by a negative CD serology (i.e., anti-transglutaminase IgA antibodies), and 150 healthy participants signed a written informed consent and were enrolled in the study.

All participants were interviewed about their usual nutritional intake during the previous year using a food frequency questionnaire (FFQ). The FFQ was administered by a trained researcher during the annual medical examination for individuals with CD at the Center of Prevention and Diagnosis of Celiac Disease. The healthy participants were interviewed at the same Center or at the Human Nutrition Unit (Department of Food and Drug, University of Parma) during a study visit. Additional data on age, date of birth, and self-reported anthropometric measures (weight, height) were collected. The local Ethical Committee for Human Research of the City of Milan approved the experimental protocols and the process for obtaining informed consent. The study was registered at ClinicalTrials.gov (ID NCT01975155).

2.2 Dietary assessment.

The electronic version of the European Prospective Investigation into Cancer and Nutrition (EPIC) FFQ developed for North-Central Italy [13], which includes 188 food items, was used to determine the usual nutritional intake of foods and beverages consumed during the previous year by healthy participants. For individuals with CD, the same FFQ, but specifically adapted for the celiac population [12], was used. Both FFQs do not ask about the frequency of intake and dosages of commonly consumed dietary supplements. On the questionnaire, the respondent indicated the number of times

a given food item was consumed (per day, week, month, or year). The participants selected an image of a food portion, or a predefined standard portion was used when no image was available to quantify portion size.
An ad-hoc computer program (Nutrition Analysis of Food Frequency Questionnaire), developed by the Epidemiology and Prevention Unit of the IRCCS Foundation, National Cancer Institute of Milan, was used to convert the questionnaire's dietary data into frequencies of consumption and mean daily quantities of foods (grams per day), energy, and nutrients consumed [13].

2.3 The Mediterranean diet score.

The adherence to an MD was evaluated by using the Italian Mediterranean Index and calculating the adherence score [5]. Scoring was based on the daily intake of the following 11 items: high intake of six typical Mediterranean foods pasta, typical Mediterranean vegetables [raw tomatoes, leafy vegetables, onion and garlic, salad, fruiting vegetables], fruits, legumes, olive oil, and fish), low intake of four non-typical Mediterranean foods (soft drinks, butter, red and processed meat, and potatoes), and alcohol. If consumption of typical Mediterranean foods is in the third tertile of the distribution, the person receives 1 point (maximum score); all other intakes receive 0 points (minimum score).
If consumption of non-typical Mediterranean foods is in the first tertile of the distribution, the person receives 1 point (maximum score). Tertiles have been calculated based on distributions of daily food intake of healthy participants. Alcohol (wine) receives 1 point for intake up to 12 g/day; abstainers and persons who consume > 12 g/day receive 0 points (minimum score). The possible scores ranged between 0 and 11 points.

2.4 Statistical analysis.

Baseline characteristics of individuals with CD and healthy participants were summarized as means and standard deviations (continuous variables) or as frequencies (categorical variables). Outliers were systematically checked, and participants for whom the ratio of total energy intake to basal metabolic rate (determined by the Harris–Benedict equation [14]) was in the first or the last percentile of the distribution were excluded to reduce the effect of implausible extreme values on the analysis. To compare the Italian Mediterranean Index score and intake of each component between individuals with CD and healthy participants, we performed analysis of covariance, with gender, age (continuous), and non-alcoholic energy intake (continuous) as covariates. Means and standard errors of the mean of the Italian Mediterranean Index and its components, adjusted for gender, age and non-alcoholic energy intake, were calculated separately for individuals with CD and healthy participants.

The *P*-values < 0.05 were considered significant. All analyses were performed with Stata version 14 (College Station, TX, USA).

3. Results

Of the 350 enrolled subjects, 66 individuals with CD and 34 healthy participants communicated to study investigators either orally or in writing that they no longer wanted to participate. Therefore, the interviewed study participants numbered 250. Of these, 23 participants were excluded since they did not provide full information on their anthropometric variables. One hundred and twenty-three individuals with CD and 104 healthy participants completed the FFQ. Of these, an individual with CD and two healthy participants were excluded from the study because of reported implausible extreme values of the ratio of total energy intake to basal metabolic rate. Therefore, the analyses were performed on 224 participants, including 122 individuals with CD and 102 healthy participants (total responders were 64%). The baseline descriptive characteristics and the mean daily reported energy intake of the study participants are presented in Table 1. The two groups of participants had similar descriptive characteristics, but individuals with CD reported a lower mean daily intake of energy than that of healthy participants.

Table 1 – Descriptive characteristics and energy intake of 122 individuals with celiac disease (CD) and 102 healthy participants who completed a study evaluating the adherence score to the Mediterranean diet using a food frequency questionnaire for recording their dietary habits[1].

	Individuals with CD (n=122)	**Healthy participants (n=102)**
Age, y	42.8 ± 13.2	38.3 ± 14.6
BMI[2],	22.6 ± 3.8	22.7 ± 3.8
Energy Intake, kcal/day	1874 ± 560	2286 ± 677
Gender[3]: Male	27 (22%)	32 (31%)
Female	95 (78%)	70 (69%)

[1]Values are mean ± standard deviation. [2]BMI= body mass index; calculated as kg/m^2.
[3]Values are n (%).

The daily reported tertile of intake for each component of the Italian Mediterranean Index used as criteria for the assignment of the maximum and minimum score is shown in Table 2.

Table 2 – Daily reported tertile of intake for each food component of the Italian Mediterranean Index of 122 individuals with celiac disease and 102 healthy participants assessed by a food frequency questionnaire. The tertile of the distribution of intake was used as criteria to assign the points of the score of adherence to a Mediterranean diet.

Food category	Food component		
		Third tertile[1]	
Typical Mediterranean Foods (g/day)	Pasta	≥ 70.2	
	Mediterranean vegetables	≥ 152.9	
	Fruits	≥ 314	
	Legumes	≥ 17.8	
	Fish	≥ 46.2	
	Olive oil	≥ 6.9	
		First tertile[2]	
Non-Mediterranean Foods (g/day)	Butter	≤ 0.2	
	Potatoes	≤ 15.3	
	Red and processed meat	≤ 52.5	
	Soft drinks	0	
	Alcohol (g/day)	0.01–12	0 >12

[1]If the consumption was in the third tertile the person receives 1 point, all other intakes receive 0 points. [2]If the consumption was in the first tertile the person receives 1 point, all other intakes receive 0 points.

To calculate the Italian Mediterranean Index score, the daily mean intake of all 11 components of the Italian Mediterranean Index was evaluated in the individuals with CD and compared to that of healthy participants (Table 3). The healthy participants had a significantly higher consumption of fruits when compared to that of the individuals with CD (P = 0.017). Conversely, no differences in the daily mean intake of the other typical Mediterranean foods considered (i.e., pasta, Mediterranean vegetables, legumes, olive oil, and fish) were observed. Regarding non-Mediterranean foods, individuals with CD consumed significantly higher amounts of potatoes (P = 0.003) and red and processed meats (P = 0.005) than healthy participants. Additionally, a higher, but not significant (P = 0.09), consumption of alcohol was reported in healthy participants compared to individuals with CD. Moreover, a lower, but not significant, consumption of soft drinks was reported in healthy participants compared to individuals with CD (P = 0.07). The resulting mean Italian Mediterranean Index was significantly higher in healthy participants than in individuals with CD (P <0.001) (Table 3).

Table 3 –Daily reported intake of single food component of the Italian Mediterranean Index and the score of adherence to the Mediterranean diet of 122 individuals with celiac disease (CD) and 102 healthy participants[1].

Food category	Food component	Individuals with CD (*n*=122)	Healthy participants (*n*=102)	*P*-value[2]
Typical Mediterranean Foods (g/day)	Pasta	49.8 ± 3.5	56.0 ± 3.9	0.249
	Mediterranean vegetables	125.9 ± 6.4	134.7 ± 7.0	0.371
	Fruits	223.5 ± 13.9	275.0 ± 15.4	0.017
	Legumes	16.3 ± 1.3	15.9 ± 1.5	0.859
	Fish	40.8 ± 2.6	41.5 ± 2.9	0.866
	Olive oil	6.1 ± 0.6	6.1 ± 0.6	0.970
Non-Mediterranean Foods (g/day)	Butter	1.3 ± 0.2	0.9 ± 0.2	0.254
	Potatoes	40.2 ± 2.9	26.8 ± 3.2	0.003
	Red and processed meat	85.4 ± 3.8	68.9 ± 4.1	0.005
	Soft drinks	55.1 ± 8.5	32.0 ± 9.3	0.075
	Alcohol (g/day)	5.5 ± 0.8	7.6 ± 0.9	0.092
Mean score		3.2 ± 0.2	4.2 ± 0.2	<0.001

[1]All the values are expressed as mean ± standard error. Reported values were adjusted for gender, age, and non-alcoholic energy intake. [2]To compare the Italian Mediterranean Index score and the intake of each component between individuals with CD and healthy participants, an analysis of covariance, with gender, age (continuous) and non-alcoholic energy intake (continuous) as covariates, was used. Significance at $P < 0.05$.

4. Discussion

In the present study, by using a score method based on the a-priori-defined Italian Mediterranean Index [5], we found that a group of Italian individuals with CD enrolled in Northern Italy, who had been on a stable diet for more than two years, had a low MD adherence score. Moreover, their average adherence score was even lower than that of healthy participants with similar descriptive characteristics.

In the last two decades, several studies have dealt with the nutritional adequacy of a GF diet. Contrasting results have been obtained from these studies, mainly due to the different dietary habits among the countries. However, researchers agree that the celiac population on the whole does not introduce the recommended amount of complex carbohydrates or fiber and they also have a low consumption of several minerals and vitamins. This population tends to have a high intake of energy from protein, saturated and total fats [9–11,15].

The reason for such poor nutritional quality of the diet has been attributed to the low quality of packaged GF foods. These foods are generally recognized as being less nutritious than their gluten-containing counterparts [16]. One of the causes of such a low nutritional quality is linked to the low palatability of these foods, which leads the food manufacturers to include more salt and lipids in their formulation [17]. It is worth to underline that the metabolic effects of using non-natural, gluten-free products on metabolic indicators, such as the glucose-insulin axis and/or the lipid profile, are only partially known [18], at short-term and even more at long-term. Furthermore, the difficulty in meeting the nutritional recommendations could also be linked to individuals simply making the wrong food choices, as already argued by Shepherd and Gibson [19].

Our results seem to mirror this hypothesis. In fact, low adherence to an MD by the individuals with CD was caused by the high daily intake of non-typical Mediterranean foods, such as potatoes and red and processed meat.

Worldwide, potatoes are often viewed as vegetables and are considered safe for individuals with CD [20]. Thus, they often replace other gluten-containing foods like pasta or bread. This aspect, along with their common use in the local traditional cuisine of Northern Italy, may have favored the higher daily consumption of potatoes observed in individuals with CD compared to the healthy participants. However, potatoes are rich in starchy carbohydrates with a high glycemic index, which may expose individuals to an increased post-prandial glycaemia [21]. Based on the results from three prospective cohort studies [22], replacing potato meals (baked, boiled, or mashed potatoes and French fries) with minor cereals and/or pseudocereals containing a high amount of fibers, such as buckwheat, quinoa, and amaranth, should be included among the dietary recommendations given to individuals with CD to protect them from the risk of type-2 diabetes.

The association between red and processed meat consumption among the world populations and NCDs risk is heterogeneous. Such heterogeneity seems attributable to several causes, i.e. the number of studies conducted and the different amounts of red and processed meat consumed among the countries [23]. However, either alone or in combination, red and processed meats are associated with a statistically significant risk of all-causes mortality [23].

According to our results from both groups of participants, the consumption of red and processed meat was high. Moreover, individuals with CD had a significantly higher consumption of these foods than healthy participants. When reported in the weekly intake, red and processed meat consumption of all individuals involved in the study surpassed the recommendation established by both the Mediterranean dietary guidelines [24] and the American Institute for Cancer Research [25]. A recent meta-analysis of prospective cohort studies suggested that total red and processed meat consumption is associated with a significant increase in the risk of all-cause, cardiovascular and cancer mortality [23]. Therefore, reduced consumption of red and processed meat should be promoted, especially among individuals with CD.

When we looked at the consumption of typical Mediterranean foods in our study, individuals with CD ate fewer fruits as compared to healthy participants. However, other typical Mediterranean foods were also consumed in low amounts by both groups. We observed a limited consumption of foods that are good sources of fiber, minerals, and vitamins, such as legumes. As a consequence, although the mean Mediterranean index score was significantly higher in healthy participants than in individuals with CD, both study groups had a low adherence to an MD. These results are in line with population-based studies that have assessed the adherence of European populations to an MD in the EPIC cohort, usually applying the Greek Mediterranean Index [26,27]. These studies showed that a high percentage of the considered population achieves low adherence scores, near to 4 points (from a total of 9). This departure from the MD has mainly been attributed to the increased consumption of red and processed meat and the reduced quantity of vegetables [27].

To date, several meta-analyses have portrayed the MD as an effective dietary pattern associated with an adequate micronutrient intake, a better health status and a lower risk of overall mortality and of developing major NCDs [4,26,28]. Therefore, it is possible to speculate that a high adherence to the MD could improve the nutritional adequacy of the GF diet of our participants and, in turn, could play a protective role in the development of major NCDs.

This study has some limitations. Firstly, the socioeconomic status and the education level of the participants were not assessed. These conditions may affect the food choices and, in turn, the dietary behavior of the study participants [29]. Secondly, only a small group of participants was investigated. However, the dietary habits were recorded using the same dietary tool (i.e., EPIC FFQ), which was validated in the general population and modified to assess the dietary habits of individuals with CD [12]. Thirdly, we used only a method to measure the MD adherence. However, to the best of our knowledge, this is the first study which evaluated the adherence to a Mediterranean diet in a group of individuals with CD who had followed a GF diet for at least two years. The method used to assess adherence to the MD — the Italian Mediterranean Index — has already been used in the Italian EPIC cohort and was able to reveal links between diet and various chronic diseases, such as the risk of stroke and cancer [5,30]. Finally, it would have been worthwhile to interview specialists and dieticians directly involved in the follow-up of CD patients in order to explore their knowledge of the

MD and its application for people suffering from CD. A pro-medicalization attitude cannot be excluded.

In conclusion, although the present findings need to be confirmed in larger population studies at a national level at least, our results bring attention to the food choices of individuals with CD and the consequent nutritional quality of their diet. These concerns are important, since there are some suggestions that the celiac condition might be associated with an increased risk of major NCDs [31,32], which may be even further increased by a low adherence to an MD.

Thus, despite the importance of the current efforts to improve the nutritional quality of GF products, whose long-term metabolic effects are largely unknown, it is necessary to encourage individuals with CD to make better food choices in line with the MD in order to improve their nutritional status and protect them from NCDs and several other health complications linked to CD. At the same time, specialists need to be made aware of the importance of this issue in order to improve the overall health of people with CD.

Conflict of interest

All authors declare that they have no conflict of interest.

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Chapter 6

Concluding remarks and future perspectives

6.1 Overview

The research activities proposed in this thesis explored several aspects of GF products and diet. Firstly, we tried to answer to what is the nutritional quality of packaged GF products, especially in light of the past and present technological improvements of product formulations (Chapter 2). Secondly, the focus was moved on the production of two common and widespread foods in the Mediterranean area, i.e., bread and pasta, was considered. Particularly, the performances of a GF system were addressed by focusing on fundamental factors as the hydration and hydrocolloids, which influences the consistency of a GF dough, and the post baking quality of GF bread. Our starting point concerned the challenge of improving the qualitative characteristics of GF products in absence of a gluten-like network. In this context, the GF bread making process was investigated from the dough mixing to the end product (Chapter 3). Thirdly, concerning pasta, the research purpose was to evaluate the potential of Chia seeds, as an innovative thickening agent, for the production of functional GF pasta with similar characteristics to those of wheat pasta (Chapter 4). Finally, moving toward a nutritional standpoint, the diet of individuals with CD was analyzed. In detail, we aimed to figure out whether the dietary habits of these individuals were healthy by scoring them with regard to MD (Chapter 5).

6.2 Summary of research methods and main outcomes

The following approaches dealt with a nutritional and technological point of view:

- Chapter 2: a score-based method was used for the nutritional analysis of Italian GF bakery products. Results highlighted a low nutritional quality of GF bakery products. Interestingly, such a low quality observed was almost similar to that of the GC counterparts.
- Chapter 3: the role of HPMC and hydration influencing the parameters describing dough consistency and bread texture was analyzed in a GF system. In detail, the synergic relationship between HPMC viscosity and hydration was addressed. We confirmed the importance of hydration as a major player in defining the consistency of a GF dough. Moreover, our results highlighted that a proper selection of HPMC relying on its (molecular-related) viscosity may help to obtain good technological features of GF dough and bread. HPMC viscosity may also help reducing the amount of HPMC used. We further observed a good and significant correlation between dough consistency and texture parameters of bread.
- Chapter 4: dietary fibers contained in Chia seeds and in exuded mucilage have interesting thickening properties. In GF pasta, both Chia seeds flour and mucilage were useful to obtain a product with similar cooking and textural characteristics to wheat pasta. Moreover, compared to

a traditional commercial GF pasta, the inclusion of Chia seeds or mucilage raised the content of protein and phenolic compounds.

- Chapter 5: a group of individuals with CD from the northern Italy was enrolled to measure how much their dietary habits were adherent to a MD. The dietary habits of these individuals were scored according to the Italian Mediterranean Index. Results evidenced that the dietary habits of individuals with CD were far from MD pattern. They had a high consumption of meat, processed meat and potatoes and a low intake of fruit and vegetables. Interestingly, such a low adherence to a MD was even lower than that of a group of healthy participants.

6.3 Scientific remarks and implications of the research activities

- Chapter 2: future research in the development of GF bakery products may take advantage of this score method, since it may represent an easy approach to evaluate their nutritional quality. It is worth stressing over importance of GF bakery products for individuals with CD. In fact, although it is true that a GF diet is also composed by naturally GF foods, the GF bakery goods replace some important GC staple foods as well as various GC products consumed for breakfast or snacking. In this view, food manufacturer could be encouraged to improve the composition of GF bakery products.
- Chapter 3: in order to better understand the importance of findings related to the synergic role of HPMC and hydration in influencing the GF bread making process, it is worth to specify the distinction between two possible production approaches. Increasing the amount of hydrocolloids (HPMC in our case) can be done up to a certain limit depending on the type of hydrocolloid. Such an approach has one of the major limitations in the cost of the ingredient. On the contrary, new research perspectives will be opened up if a physicochemical feature of the hydrocolloid molecule as the viscosity, which is related to the molecule chain length, is used to influence the consistency of a dough and then the bread texture. The (molecular-related) viscosity of HPMC, scarcely considered so far in bread making, may allow the reduction of the amount of hydrocolloids and the optimization of a GF bread formulation also in view of future formulation enrichments.
- Chapter 4: future research in innovation of GF cereal products cannot disregard the nutritional importance of the selected ingredients. Nowadays, a particular tendency towards the preference of functional ingredients or *superfoods* is influencing the food innovation sector of both GF and GC products. Individuals with CD, as the general population, are influenced by nutrition trends, i.e., those referred to food products with a short list of ingredients and/or reduced presence of additives perceived as non-natural. Chia seeds gained importance because they can represent the

idea of an ancient and natural food. Moreover, Chia seeds are also considered a *superfood* because of its high content of dietary fibers, phenolic acids, vitamins and minerals.

- Chapter 5: some dietary habits, both for individuals with CD and for the general population, can pose a health risk. For instance, it is known that the westernization of dietary habits and the consequent detachment from healthy dietary models, such as the MD, leads to an increased number of overweight and obese people and increases the risk of major NCDs. To date, there are not enough evidences supporting the role of CD itself in rising the risk of major NCDs for individuals with CD with respect to the non-celiac population. Causes of such dietary habits of individuals with CD can be multiple. For instance, the focus on gluten exclusion and the several restrictions imposed by the GF diet. However, the information collected should sensitize the scientific community and the healthcare professionals during the nutritional counseling to individuals with CD.

6.4 Future perspectives

Notwithstanding the results obtained are encouraging, further research should contemplate some of the following considerations:

Chapter 2: the number of GF products is prone to a continuous growth due to the innovations in food industry, the increased variety of ingredients available and a constant demand from consumers for new products. Further integration of the developed score with information about micronutrients content of GF bakery products would be useful to allow a more comprehensive nutritional evaluation.

Chapter 3: the optimization of a simple formulation for producing GF bread obtained with a proper inclusion of HPMC and the modulation of the hydration level may open up several production perspectives. Indeed, the reached stability of the GF system may support the addition of functional ingredients, as inulin-type fructans. Inulin-type fructans are actually known for their contribution to the overall dietary fibers content of GF bread and for their prebiotic activity.

Chapter 4: since Chia seeds carry several important bioactive compounds, such as phenolic compounds and dietary fibers, it may be interesting to deepen the fate of these molecules during digestion. Indeed, it is known that the amount of certain phenolic compounds may decrease following the digestion process. Furthermore, little is known about Chia seeds fiber prebiotic activity. Therefore, the influence of these seeds on intestinal bacteria dynamics and production of healthy relevant metabolites as SCFA should be investigated.

Chapter 5: results obtained from the nutritional evaluation of the GF diet of individuals with CD poses some interesting questions that deserve more attention. However, our study is limited to a small group of people and to a specific Italian geographical area. Indeed, several local dietary habits may differentially affect the dietary choices of individuals with CD. Therefore, also the rate of adherence to a MD may undergo variations according to different geographical areas. To deal with such aspects, the analysis of the dietary habits should be performed by enrolling patients from different Italian regions.

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Federico Morreale has a Master Degree in Biology at University of Parma.

After graduation he started working at the Department of Food Science with the group of Food Microbiology led by professors Erasmo Neviani and Monica Gatti. Working with food microbiologist and other food scientists he earned interest in the food sciences field. Thus, after obtaining a PhD fellowship in Food Science and Human Nutrition, he started collaborating with Professor Nicoletta Pellegrini on a research project on the quality aspects of gluten-free products and diet.

During the PhD course he worked with medical professional in human nutrition, gastroenterology and immunology to set up a survey on the dietary habits of Italian individuals with celiac disease, thus continuing a previous study of Dr Teresa Mazzeo. The main idea was to acquire nutritional information about the gluten-free diet to verify whether individuals with celiac disease had a balanced dietary intake.

At the same time, he deepened the technological aspects of gluten-free products by focusing on the role of hydrocolloids and other ingredients rich in dietary fiber. Regarding this, he spent eight months at the Institute of Agrochemistry and Food Technology (IATA-CSIC) of Valencia. There, under the supervision of professor Cristina Molina Rosell, his main activity was about the innovation strategies in gluten-free product production aimed at having more palatable and nutritious foods. Therefore, by using the facilities made available by the Institute, he explored the role of hydroxypropylmethylcellulose as thickening in agent in bread, and tried to understand the main players in fructans loss during the bread production process.

Future perspectives of his research activity will concern the food sciences and the innovation field.

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