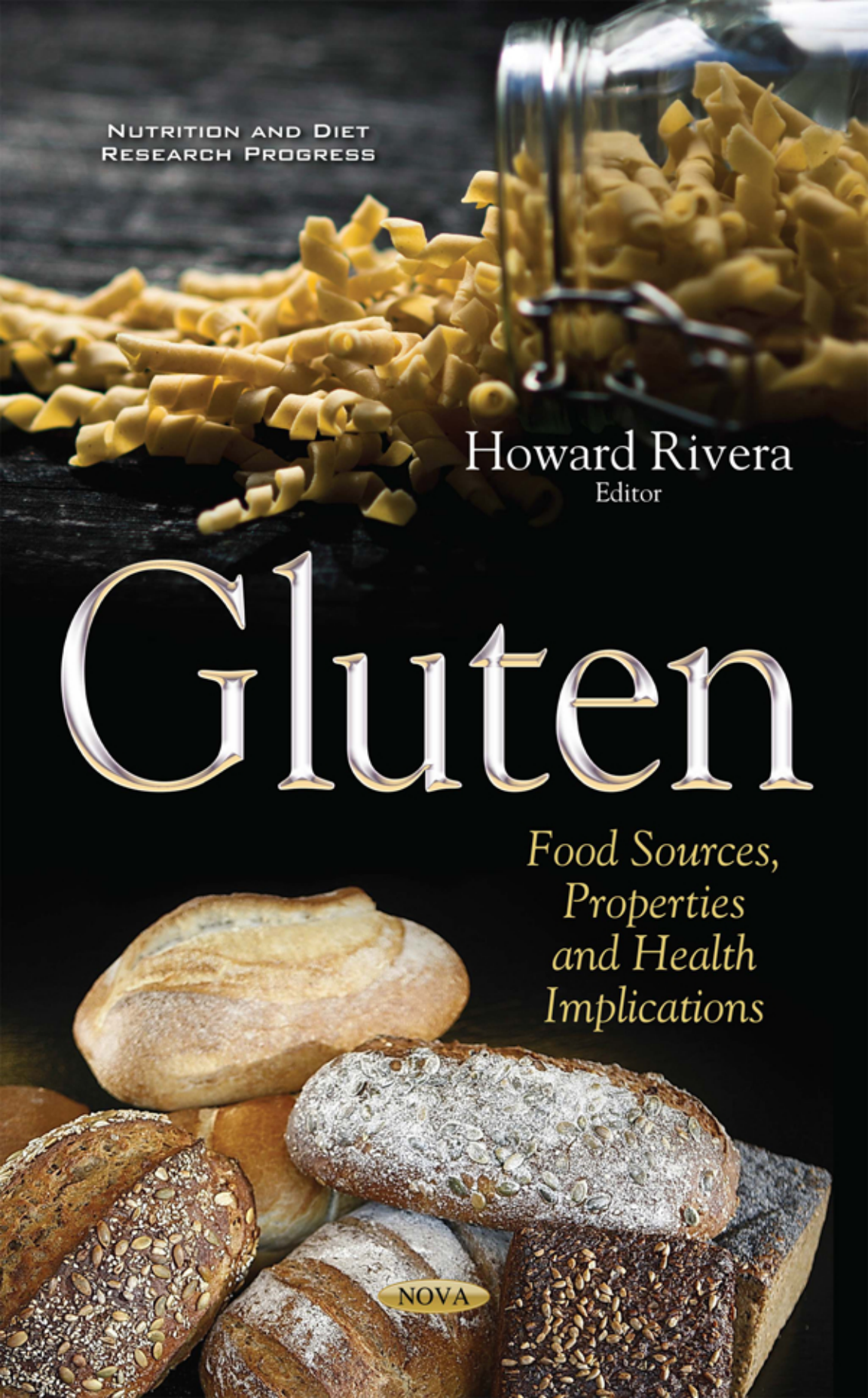
Nutrition and Diet
Research Progress
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NOVA

NUTRITION AND DIET RESEARCH PROGRESS

GLUTEN

FOOD SOURCES, PROPERTIES AND HEALTH IMPLICATIONS

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NUTRITION AND DIET RESEARCH PROGRESS

GLUTEN

FOOD SOURCES, PROPERTIES AND HEALTH IMPLICATIONS

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EDITOR

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CONTENTS

PREFACE

Gluten and gluten-related proteins (prolamin and glutelin) may be present in several cereals, such as wheat, rye, barley, oat and the derivatives of these grains, including malt and brewer's yeast. Despite of some specific health implications, cereals are important carbohydrate and proteins source for human diet. Phenolic acids, vitamins, minerals and dietary fiber also can be found in wholegrains. Nowadays, cereals have been investigated about its potential use as ingredient in functional foods. Therefore, the development of food products with health benefits is a challenge for the food industry. This book provides new research on gluten's food sources, properties and health implications.

Chapter 1 - Gluten is composed of monomeric gliadins and polymeric glutenins and is considered to be the main source of the viscoelastic properties of wheat dough. The United Nations declared that *2016* is the *International Year of Pulses*. Owing to their amino acid composition and fiber content, pulse flours are ideal ingredients for improving the nutritional value of sweet baked products. The effect on rheological properties of muffin batter of replacing wheat flour (WF) with chickpea flour (CF) was studied by using oscillatory (including gelatinization kinetics), creep and recovery, and steady-state shear (considering time dependence) tests. CF was used to replace WF in the batter partially (25, 50, 75% w/w) or totally (100% w/w, i.e., CF-based gluten-free muffin batter), and compared with a control made only with gluten (100%WF batter). 100%WF batter, with higher structural stability on short time scales, can be characterized as a weak gel, while batters with partial and total WF replacement presented a weaker structure. However, batters with 0 and 100% replacement levels (gluten and free-gluten batters, respectively) had similar gel points. Zero-order reaction kinetics described the batter gelatinization process

well, with activation energies ranging between 113.43 and 204.38 kJ mol^{-1}. The lower activation energy (113.43 kJ mol^{-1}) of 100%WF batter implies that it was more favorable for gelatinization. Creep and recovery tests showed that the gluten WF batter had the lowest compliances during creep and recovery stages meaning that gluten batter was denser and most time-stable than the CF-based batters. Under viscous shear flow, 100%WF batter was the most thixotropic, with the lowest viscosity recovery percentage and resistance to flow, the longest rebuild time, and the highest fluidity. Therefore, elasticity, extent of thixotropy, and rebuild time (from viscosity measurements) decreased significantly with increase in WF replacement level. Steady flow data fitted the Herschel–Bulkley model well. Complex and apparent viscosities failed to follow the Cox–Merz rule.

Chapter 2 - Coeliac disease is a permanent intolerance to gluten proteins of many common cereals such as wheat, rye, barley and oat. Therefore, coeliac patients must be on a strict long-life gluten-free diet, which is usually poor in some essential nutrients. Due to the limitation of some nutrients, the fortification of gluten-free products is required to obtain a balanced diet for coeliac patients. A growing number of studies have investigated the application of pseudocereals in the production of nutrient-rich gluten-free products such as bread, pasta and confectionery products.

This paper offers the overview of application of one of the most used pseudocereals in gluten-free bakery and confectionery formulations – buckwheat grain. It refers to the application of light and wholegrain buckwheat flour in different formulations to obtain added value products with described reological characteristics of dough and nutritional and functional benefits and sensory parameters of the final products. A number of different types of buckwheat-containing gluten-free bread was chosen as the most frequently used bakery products to be descriebed, as well as buckwheat-containing gluten-free cookies and creckers as the representatives of confectionery products.

Chapter 3 - Celiac disease (CD) is an autoimmune disorder, occurring in genetically predisposed individuals, triggered by the ingestion of dietary gluten, the major protein component in wheat and other related cereals. In many areas of the world, CD is one of the commonest lifelong disorders affecting around 1% of the population. Indeed, CD research is changing rapidly as gluten-related disorders have gradually emerged as an epidemiologically relevant phenomenon with a global prevalence. Among such disorders, CD and wheat allergy (WA) have been extensively studied although they are not the only entities, as non-celiac gluten sensitivity (NCGS)

has been recently re-discovered and appears to be a very common disorder, in particular in the U.S.A.

A lifelong gluten-free diet (GFD) is currently the only available treatment for such disorders. Clinical manifestations associated with untreated patients, including intestinal but also extra-intestinal manifestations are ameliorated with a GFD. However, despite a strict adherence to the diet is essential to reduce symptoms, estimated compliance rates vary considerably in CD (17-80%). Despite the importance of monitoring the GFD, there are no clear guidelines for assessing the outcome or for exploring its adherence. Available methods are insufficiently sensitive to detect occasional dietary transgressions that may cause gut mucosal damage. Thus, detection of gluten immunogenic peptides (GIP) in feces and urine has been proposed as new non-invasive biomarker to determine gluten intake and monitor GFD compliance in patients with CD. This method has showed high sensibility and significant correlation with consumed gluten, enabling assessment of gluten exposure early after ingestion and could aid in the diagnosis and clinical management of non-responsive CD and refractory CD.

Chapter 4 - Gluten is a specific combination of protein that is present in wheat, rye, oats, barley, and their derivatives, such as flour, bread, cakes, and biscuits. This combination consists of two protein fractions called prolamins and glutenins. Prolamins are given different names according to their origin: gliadin in wheat, secalin in rye, and hordein in barley. Prolamins are the cause of the allergic reaction characteristic of celiac disease. Celiac disease is the result of genetically susceptible individuals allergic to gluten, causing inflammatory damage and abnormal intestinal mucosa. Therefore it is an autoimmune disease. Because of the effects that gluten causes in some patients, foods that contain gluten have been viewed with suspicion and wrongly removed from the diet, even when it comes to healthy individuals who have no sensitivity or allergy to gluten. The aim of this chapter is to explain and clarify doubts about gluten and its effects in the body, both in celiac patients and in those without the disease.

Chapter 5 - Gluten and gluten-related proteins (prolamin and glutelin) may be present in several cereals, such as wheat, rye, barley, oat and the derivatives of these grains, including malt and brewer’s yeast. Consequently, several commercial foods contain gluten proteins in its composition, for example: pastas, cookies, breads, gravies, salad dressings, soups, processed lunchmeats and snack foods. Despite of some specific health implications, cereals are important carbohydrate and proteins source for human diet. Phenolic acids, vitamins, minerals and dietary fiber also can be found in wholegrains.

Nowadays, cereals have been investigated about its potential use as ingredient in functional foods. Therefore, the development of food products with health benefits is a challenge for the food industry. The consumers demand for food with sensorial quality and suitable to specific restrictions have encouraged the diversification of general cereal-based foods intake.

Gluten is the main structure-forming protein, primarily in wheat flour, composed for two different proteins: gliadin (a prolamin fraction) and glutenin (a glutelin fraction). An acceptable definition for the protein complex is "cohesive, viscoelastic proteinaceous material" that remains when wheat dough is washed to remove starch granules and water soluble constituents. Focused on wheat flour proteins, monomeric gliadins and polymeric glutenins (*high molecular weight glutenin subunit* - HMW-GS and *low molecular weight glutenin subunit* - LMW-GS) impart distinct functions on gluten formation, depending on capable to form intra- and inter-chain disulfide bonds. The properties of gluten become apparent when flour is hydrated, giving elastic and viscous characteristics to dough, with good gas holding properties, and a good crumb structure in baked bread. Gluten is often termed the 'structural' protein for breadmaking being largely responsible for end-use quality of wheat in many food products. The absence of gluten results in major problems for bakers, and currently, many gluten-free products available on the market are of low quality, exhibiting poor mouthfeel, texture and flavor.

Celiac disease (CD) is the main health gluten implication and the most common food induced enteropathy in humans. CD is defined as an inherited autoimmune disorder that affects the digestive process of the small intestine that develops in predisposed subjects, and is triggered by ingestion of gluten. Celiac disease is a life-long intolerance to the gliadin fraction of wheat and the prolamins of rye (secalins), barley (hordeins) and possibly oats (avidins). Celiac disease affects 1 in 100-200 individuals, this prevalence is significantly higher than that recognized years ago. The effective treatment for celiac disease is a gluten-free diet throughout one's lifetime, which results in clinical and mucosal recovery, when followed closely. However, patients can present other implications that are not celiac disease as allergy to gluten which activates the IgE (immunoglobulin E) and consequently histamine, mast cells and basophils, causing the allergic attack. A change in intestinal permeability gives rise to the passage of antigens which leads the onset of diseases including those related to gluten.

Chapter 6 - Celiac disease is a chronic immune-mediated enteropathy triggered by the ingestion of gluten in genetically-predisposed individuals.

Treatment of celiac disease is based on life-long adherence to a gluten-free dietary regimen.

Some celiac patients experience persistence or recurrence of symptoms after a period of well-being, despite an ongoing gluten-free diet. This condition, defined as non-responsive celiac disease, affects nearly one fifth of celiac patients and may be due to heterogeneous etiologies. Its occurrence warrants a correct assessment in order to optimize the management of celiac patients.

This chapter focuses on available evidence on frequency and cause of non-responsive celiac disease, and on suggested investigations for the correct assessment and management of this condition.

The initial diagnosis of celiac disease should be reconsidered. In patients with confirmed diagnosis of celiac disease, a diet compliance assessment is among the first and mandatory steps to be undertaken, to differentiate non-responsive celiac disease from refractory celiac disease, as the latter carries a significantly different burden of clinical implications. Further laboratory tests, breath-tests, endoscopic and histologic evaluations are warranted according to the persistent symptoms/signs in order to identify alternative or concomitant disease presenting with symptoms which overlap those of celiac disease.

Once the cause of non-responsive symptoms has been identified it should be targeted accordingly in order to achieve symptoms improvement or resolution.

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Chapter 1

A COMPARISON OF GLUTEN WHEAT FLOUR FUNCTIONALITY VERSUS GLUTEN-FREE CHICKPEA FLOUR, AND THEIR MIXTURES, IN THE OSCILLATORY, TRANSIENT, AND STEADY RHEOLOGICAL PROPERTIES OF MUFFIN BATTERS

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ABSTRACT

Gluten is composed of monomeric gliadins and polymeric glutenins and is considered to be the main source of the viscoelastic properties of

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wheat dough. The United Nations declared that *2016* is the *International Year of Pulses*. Owing to their amino acid composition and fiber content, pulse flours are ideal ingredients for improving the nutritional value of sweet baked products. The effect on rheological properties of muffin batter of replacing wheat flour (WF) with chickpea flour (CF) was studied by using oscillatory (including gelatinization kinetics), creep and recovery, and steady-state shear (considering time dependence) tests. CF was used to replace WF in the batter partially (25, 50, 75% w/w) or totally (100% w/w, i.e., CF-based gluten-free muffin batter), and compared with a control made only with gluten (100%WF batter). 100%WF batter, with higher structural stability on short time scales, can be characterized as a weak gel, while batters with partial and total WF replacement presented a weaker structure. However, batters with 0 and 100% replacement levels (gluten and free-gluten batters, respectively) had similar gel points. Zero-order reaction kinetics described the batter gelatinization process well, with activation energies ranging between 113.43 and 204.38 kJ mol^{-1}. The lower activation energy (113.43 kJ mol^{-1}) of 100%WF batter implies that it was more favorable for gelatinization. Creep and recovery tests showed that the gluten WF batter had the lowest compliances during creep and recovery stages meaning that gluten batter was denser and most time-stable than the CF-based batters. Under viscous shear flow, 100%WF batter was the most thixotropic, with the lowest viscosity recovery percentage and resistance to flow, the longest rebuild time, and the highest fluidity. Therefore, elasticity, extent of thixotropy, and rebuild time (from viscosity measurements) decreased significantly with increase in WF replacement level. Steady flow data fitted the Herschel–Bulkley model well. Complex and apparent viscosities failed to follow the Cox–Merz rule.

Keywords: gluten, wheat flour, gluten-free, chickpea flour, muffin batter, viscoelasticity, creep-recovery, thixotropy, microstructure

INTRODUCTION

A muffin batter may well be defined as a “cellular system” [1], in which the continuous semi-solid matrix formed by a complex fat-in-water emulsion could be considered a multi-phase system containing ungelatinized starch granules, oil droplets, proteins, sucrose, etc. Moreover, those “cells” are filled with air bubbles as the discontinuous phase. In turn, baked muffins are characterized by a typical alveolar-porous structure and high volume, which

give a spongy texture. To obtain this final structure, a stable semi-solid matrix lodging many tiny air bubbles is required [2].

Traditionally, a muffin recipe is mainly composed of WF, sucrose, vegetable oil, egg, and milk [3]. For this reason, people with celiac disease are unable to consume this type of baked product. In recent years, there has been extensive research for the development of gluten-free bakery products. Nevertheless, the manufacture of baked products without gluten presents bakers with major technological problems. In fact, many gluten-free products available on the market are often of poor technological quality, exhibiting low volume, poor color, and crumbling crumb, as well as great variation in nutrient composition, with low protein and high fat contents [4], particularly when compared with their wheat counterparts [5]. Commercially manufactured gluten-free muffins should aim to resemble those made from WF, overcoming problems of quality defects and low nutritional value.

Most of gluten -free muffin, cake, or cupcake recipes contain rice flour as the principal ingredient, or other starch sources, such as rice, corn, potato, and sorghum [6]. Additionally, the incorporation of dairy proteins has long been established in the bakery industry, and legumes such as soybean can also be good supplements for cereal-based foods because they increase the protein content and complement the nutritional value of cereal proteins [5]. Nevertheless, other legumes are rich sources of protein throughout the world and contain approximately three times more protein than cereals. Chickpea (*Cicer arietinum* L.) is one of the top five important legumes on the basis of whole grain production [7]. The potential for increased use of chickpea is related to its relatively low cost, relatively high protein content (~18.3–28.9%), high protein digestibility (76–78%), and other desirable functionalities [8].

Therefore, chickpea flour (CF) could be an excellent choice to improve the nutritional value of gluten-free or partially replaced wheat flour (WF) in muffin batter. The high lysine and low methionine contents complement those of WF proteins, which are poor in lysine and relatively higher in the sulfur-containing amino acids [7]. According to the authors just cited, studies have shown that CF can be successfully incorporated into products at up to 20% inclusion, to produce products that rate higher in terms of color, texture, taste, and overall acceptability. A number of pasta products containing CF are currently available on the domestic market. Also, CF has been incorporated in biscuits at a level of up to 50% [9]. On the other hand, the United Nations has declared that 2016 will be the International Year of Pulses named as “IYP

2016." The hope of "IYP 2016" is to position pulses as primary sources of protein and other essential nutrients, leading to dietary uptake.

Small-amplitude oscillatory shear (SAOS) and creep-recovery are fundamental tests that measure the linear viscoelastic properties of a substance. The stress applied to the sample in the linear viscoelastic (LVE) region is low enough not to produce an irreversible change in the structure, so information about the unaltered system structure is obtained [10]. Flow and its characterization during pumping and handling of muffin batters are very important because the batter is an intermediate product that subsequently passes through low (mixing operations) and high (pumping) shear operations. Therefore, a complete rheological characterization of polysaccharide systems entails viscosity and viscoelastic measurements. SAOS measurements are considerably easier to perform than steady-state shearing experiments, and therefore the analysis of the applicability of the Cox–Merz rule is always of interest.

The aim of this work was to evaluate the suitability of CF to replace different percentages of WF in Spanish muffins, and to study its functionality in the linear viscoelastic properties (oscillatory and creep and recovery tests) and the flow behavior (steady-state shear tests) of the batter as a prior step to evaluating and understanding other properties (texture and sensory acceptability) of the baked muffin. Optical microscopy of batter was also analyzed, and conclusions about the structural features associated with WF replacement were drawn.

MATERIALS AND METHODS

Batter Preparation

Five muffin formulations (Table 1) were prepared by replacing part or all of the wheat flour (WF) with chickpea flour (CF). The Spanish muffin ingredients were: wheat flour (Triticum spp. with 13.5% moisture and 10.2% protein content as specified by the supplier) and chickpea (C. arietinum 'Castellano') flour, both donated by the García del Valle flour milling company (Soria, Spain), pasteurized liquid whole yolk (Ovopak®, Alvarez Camacho S.L., Seville, Spain), sucrose (AB Azucarera Iberia S.L., Madrid,

Spain), salt (sodium chloride), ultra-high temperature whole milk (Pascual, Burgos, Spain), refined sunflower oil (Koipesol, Madrid, Spain), natural lemon juice (cultivar 'Primofiori', Spain), and sodium bicarbonate (Montplet and Esteban, S.A., Madrid, Spain), in accordance with the method used previously by other authors Martínez-Cervera et al [2]. Mean values for proximate composition (g 100 g^{-1}) of chickpea flour, as specified by the supplier, were as follows: moisture, 14, crude fiber, 15, crude protein (N × 6.25), 19.4, fat, 5, carbohydrate, 55, and energy, 330 kcal 100 g^{-1}. The samples were identified as 100%WF (control gluten batter), 25%CF, 50%CF, 75%CF, and 100%CF (Table 1).

Table 1. Formulations of control muffin batter prepared with 100% of wheat flour (WF) and batters prepared with increasing quantities of chickpea flour (CF) as WF replacer

Ingredients (g 100 g^{-1} flour)	100%WF	25%CF	50%CF	75%CF	100%CF
WF	100	75	50	25	0
CF	0	25	50	75	100
Whole egg	81	81	81	81	81
Sucrose	100	100	100	100	100
Salt	0.75	0.75	0.75	0.75	0.75
Milk	50	50	50	50	50
Oil	46	46	46	46	46
Natural lemon juice	3	3	3	3	3
Sodium bicarbonate	4	4	4	4	4

The batter was prepared in a KPM5 professional mixer (Kitchen Aid, St. Joseph, Michigan, USA), in which the egg was whisked for 2 min at top speed (speed 10, 220 rpm), and the sucrose was added and mixed for 30 s. Then the mixer speed was reduced to 4, and lemon juice and half the milk were added and mixed for 1 min. The flour, sodium bicarbonate, and sodium chloride were added and the mixture was beaten for a further 1 min. Lastly, the mixer speed was increased to 6, the rest of the milk was added, and the oil was gradually dripped in. The mixture was beaten for 3 min until it was smooth. Each formulation was prepared at least six times (six batches), on different days, to carry out the various rheological tests.

Rheological Properties of Muffin Batter

All rheological measurements, SAOS and steady shear tests, were performed using either a Bohlin CVR 50 controlled stress rheometer (Bohlin Instruments Ltd., Cirencester, UK) and a Kinexus pro rotational rheometer (Malvern Instruments Ltd, Worcestershire, UK), using parallel-plate geometry (40 mm diameter and 1 mm gap). The batters were all kept at 25°C for 60 min after batter preparation before the rheological measurements. They were then put on the lower plates of the rheometers for measuring. Any excess sample protruding beyond the upper plate was carefully removed. Samples were allowed to rest for 15 min before analysis to ensure both thermal and mechanical equilibrium at the time of measurement. Samples were covered with a thin film of Vaseline oil (PRS-Codex) to avoid evaporation. The observed adherence between the measuring system and the sample was sufficient to transmit the shear stress, therefore there was no slippage. The temperature was controlled to within 0.1°C by Peltier elements in the lower plates kept at 25°C, except when non-isothermal heating processes were carried out. Three replicates of each test were run with samples prepared on three different days. Results are means of nine replications from three batches of each formulation.

SAOS Measurements

Stress Sweep Tests

To determine the LVE region, stress sweeps were run at 6.28 rad s^{-1} (1 Hz) at 25°C with the shear stress (σ) of the input signal varying from 0.01 to 100 Pa. Forty-one points on the continuous mode were measured in all instances. Changes in storage modulus (G', Pa), loss modulus (G'', Pa), complex modulus (G^*, Pa), and loss tangent ($\tan \delta = G''/G'$, dimensionless) were recorded. The critical (maximum) values of shear strain (γ_{max}) and shear stress (σ_{max}) at the limit of the LVE range were obtained according to the method previously described by [11].

Frequency Sweep Tests

Samples were subjected to stress that varied harmonically with time at variable frequencies from 0.01 to 100 Hz (~0.06 – 62.83 rad s^{-1}). The strain amplitude was set at $\gamma = 0.1\%$, within the LVE range.

Temperature Sweep Tests

Temperature sweep tests were performed from 25 to 125°C at a linear heating rate (1.6°C min^{-1}). The oscillation frequency was set at 1 Hz, but maintaining the σ signal at the minimum value provided by the Bohlin rheometer for parallel-plate geometry (2.98 Pa). For this stress, the corresponding strain amplitude was higher than 0.1%; therefore, temperature sweeps were carried out outside the LVE range until starch gelatinization process occurred in order to obtain the gel points (cross-over of elastic modulus, G' and viscous modulus, G'') of the batters.

Creep and Recovery Tests

An instantaneous stress σ_0 ranging between 0.08 and 0.16 Pa for the different samples, depending on the replacement percentage, and corresponding to 0.1% shear strain within the LVE range, was applied for 600 s in the creep tests and the resulting change in strain over time $\gamma(t)$ was monitored. When the stress was released, some recovery was also observed for 600 s. The creep and recovery results were described in terms of the shear compliance function $J(t) = \gamma(t)/\sigma_0$. Compliance curves generated at different linear stress levels overlap, making it possible to examine and compare the structural properties of the different food gels on larger time scales [12].

Steady Shear Rheological Measurements

Flow Time Dependence and Flow Behavior

To study flow time dependence, the hysteresis loop was obtained by recording shear stress at shear rates from 0.1 to 100 s^{-1} in 5 min and down in 5 min. Areas under the upstream data points (A_{up}) and under the downstream data points (A_{down}) as well as the hysteresis area ($A_{up} - A_{down}$) were obtained using Bohlin CVO 120 software (v. 06.40).

Before analyzing flow behavior, the structure responsible for thixotropy was previously destroyed by shearing [13]. After testing several shearing conditions, for 5 min at 100, 200, and 300 s^{-1} shear rates, and analyzing the hysteresis cycles, a previous shearing of 5 min at 300 s^{-1} was selected as the appropriate treatment to obtain a reduced hysteresis area. After eliminating or reducing the flow time dependence, sample flow was again measured by controlling the shear rate and recording the shear stress values at shear rates from 0.1 to 100 s^{-1} in 5 min and down in 5 min.

Three-Step Shear Rate Tests

For viscometry rebuild analysis, the samples were subjected to a shear rate of 0.1 s^{-1} for 30 s in the first stage. Then, in the second stage, a shear rate of 100 s^{-1} was applied for 30 s in order to imitate the breakdown of the sample's structure. In the third stage, the shear rate was again dropped to 0.1 s^{-1} and the viscosity recovery was monitored for 600 s. Then, the percentage of viscosity recovery at the end of the test relative to the original apparent viscosity values was calculated form the original (η_0) and the final apparent viscosity (η_f) values as $(\eta_f \times 100)/\eta_0$. Both, the percentage of viscosity recovery and the time (s) taken in the third stage (after structural breakdown) to achieve rebuild of the viscosity to 90% of the original value were considered as reliable measurements of thixotropy.

Color and Other Physical Properties of Muffin Batter

The color of the muffin batter in pots was measured with a Hunter-Lab model D25 (Reston, VA) color difference meter fitted with a 5-cm-diameter aperture. Results were expressed in accordance with the CIELAB system with reference to illuminant D65 and a visual angle of 10°. The parameters measured L^* ($L^* = 0$ [black], $L^* = 100$ [white]), a^* ($+a^*$ = red) and b^* ($+b^*$ = yellow).

The total color difference (ΔE^*) between the control sample and each of the batters was calculated (Eq. 1; [14]:

$$\Delta E^* = \left((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\right)^{1/2} \quad (1)$$

The values used to determine whether the total color difference was visually obvious were the following [15]:

$\Delta E^* < 1$: color differences are not obvious for the human eye;

$1 < \Delta E^* < 3$: minor color differences could be appreciated by the human eye, depending on the hue;

$\Delta E^* > 3$: color differences are obvious for the human eye.

The specific gravity (SG) of the batter was measured as the ratio of the weight of a standard container filled with batter (W_2) to that of the same container filled with water (W_1) in accordance with [2].

The total soluble solids (TSS) content [g $100g^{-1}$ (w/w)] as measured by refractive index and the pH of the batters were determined with an Atago (Itabashi, Tokyo, Japan) dbx-30 refractometer and a Schott CG pH meter (Model 149 842; Schott-Geräte GmbH, Mainz, Germany), respectively.

Water content (%) was determined by drying samples in a Philips microwave oven (model M-718, 700W) with output power at 70%. In all cases, three different batches were employed and each formulation was measured in triplicate.

Optical Microscopy

The muffin batter samples were imaged using an Optiphot SMZ microscope (Nikon, Japan) with a magnification of 10× in clear field mode. Baked muffins were observed using an L2-S8 APO Stereomicroscope (Leica Microsystems, Wetzlar, Germany) with a magnification of 1.6×. In both cases, the micrographs were taken with a Nikon Digital Coolpix 5000 color digital camera. Each formulation was prepared twice, on different days, and four replicates of each batter and baked muffin were photographed.

Statistical Analysis

For each property or parameter evaluated, one-way analysis of variance (ANOVA) was carried out by using the SPSS® Statistics 19.0 computer program (SPSS Inc., Chicago, IL, USA). Bonferroni's multiple comparison procedure was used to assess significant differences ($P < 0.05$) among batters that might allow discrimination between them.

RESULTS AND DISCUSSION

SAOS Measurements of Muffin Batter

Stress Sweep Tests

Stress sweeps were carried out to determine the influence of WF replacement within the limits of the LVE range. For this purpose, stress (σ_{max}) and strain (γ_{max}) amplitudes, complex modulus (G^*), and loss factor (tan δ)

were used to limit the LVE range (Table 2). These critical σ_{max} and γ_{max} values can be taken as measurements of rheological stability [16] and they were obtained by defining the range of tolerable deviation as 10% [11]. This means that all G^* below 90% of the plateau value are considered to be outside the LVE range. Both G' and G'' were also obtained to limit the LVE range, showing gel behavior with $G' > G''$ throughout the complete LVE range only for the control gluten batter without added CF.

Table 2. Effect of wheat flour replacement on limit values of linear viscoelastic (LVE) range from stress sweep tests of the muffin batter at 25°C

Type of formula	σ_{max} (Pa)	γ_{max} (%)	G^* (Pa)	tan δ (-)
100%WF	0.642±0.01^{d}	0.63±0.03^{c}	102±5.3^{a}	0.86±0.05^{b}
25%CF	0.646±0.01^{b}	0.96±0.06^{a}	68±4.2^{c}	1.05±0.06^{a}
50%CF	0.644±0.01^{c}	0.81±0.08^{a,b}	80±8.0^{b,c}	1.06±0.15^{a}
75%CF	0.643±0.01^{c,d}	0.76±0.11^{b,c}	86±11.8^{a,b}	1.03±0.08^{a,b}
100%CF	0.808±0.00^{a}	0.84±0.03^{a,b}	96±3.3^{a,b}	1.09±0.03^{a}

Values are given as mean (n = 9) ± standard deviation. $^{a-d}$ Different letters in the same column mean significant differences ($P < 0.05$) among samples according to Bonferroni multiple range test. WF: wheat flour; CF: chickpea flour; σ_{max}: shear amplitude; γ_{max}: strain amplitude; G^*: complex modulus; tan δ: loss tangent = G''/G'.

Notably, after total WF replacement (100%CF) there was a very significant increase in σ_{max} with respect to the 100%WF control gluten batter, whereas γ_{max} was similar to the rest of the samples with added CF. Batters prepared with increasing quantities of CF replacing WF had higher γ_{max} values than the gluten sample (100%WF). This means that the batters with CF at 25–100% levels were more deformable and had a higher degree of conformational flexibility [17] than the control gluten batter, as also evidenced by their lower critical G^* and higher tan δ values, reflecting the loss of rigidity and also of elasticity (high tan δ) of the batters as WF was replaced by CF. The unique bread-making properties of WF can be attributed mainly to the ability of its gluten proteins to form a viscoelastic structural system when mixed with water [7]. The results also indicate that the reinforcement of the physical structure of control gluten batter is associated with a narrowing of the LVE range.

Therefore, 100%WF batter was more rigid (high G^*) and brittle, i.e., more shear sensitive (lower σ_{max} and γ_{max}).

In particular, although there were no statistically significant differences between the tan δ values of the batters prepared with increasing quantities of CF, tan δ was highest in the 100%CF sample. This lower viscoelasticity reveals a less elastic character of the structural system as the WF was replaced by CF. Note that for all the batters formulated with CF at any percentage the tan δ values were >1, indicating an increase in the predominance of the viscous component versus the elastic component below the limiting value (γ_{max}), especially in the case of chickpea-based gluten-free muffin batter. Thus, it seems that the gluten produced a structural improvement in the structure of the batter showing this control gluten batter a more solid-like character (tan δ < 1) (Table 2). Protein from chickpea contains 11S legumin and 7S vicilin as the major protein fractions [18]. This relatively high globulin content of chickpea could be responsible for the lower rigidity of the structure as the WF is increasingly replaced by CF.

Frequency Sweep Tests of Muffin Batters

The influence of partial or total WF replacement with CF on the frequency sweeps carried out in the linear region are shown in Figure 1. The mechanical spectra of the five batters are apparently quite similar, although some differences can be appreciated. In the control gluten batter (100%WF), the behavior was predominantly more elastic because storage moduli (G') was greater than loss moduli (G'') in the entire frequency (ω) range studied. This could be clearly seen from tan δ (G''/G') values (data not shown) because tan δ was less than 1, ranging between 0.65 and 0.91, in the entire ω range. On the other hand, differences between both moduli (G' and G'') decreased at both lower and higher ω. In turn, in both formulations prepared with the higher CF levels (75%CF, and 100%CF) G' was also greater than G'', although only in the ω range from 0.1 to 10 rad s^{-1}. In the 75%CF and 100%CF batters, for ω < 0.1 rad s^{-1} and ω > 10 rad s^{-1}, G'' was greater than G', reflecting a viscous behavior at both low and high frequencies. For batters containing the lower CF levels (25%CF and 50%CF), the behavior was predominantly more elastic at intermediate and high ω, but tan δ was also more than 1 at low ω. Therefore, control gluten batter can be characterized as a soft or weak gel, while the samples with partial and total WF replacement presented a structure between those of a concentrated biopolymer and of a weak gel, with tan δ values ranging between 0.80 and 1.41 [19].

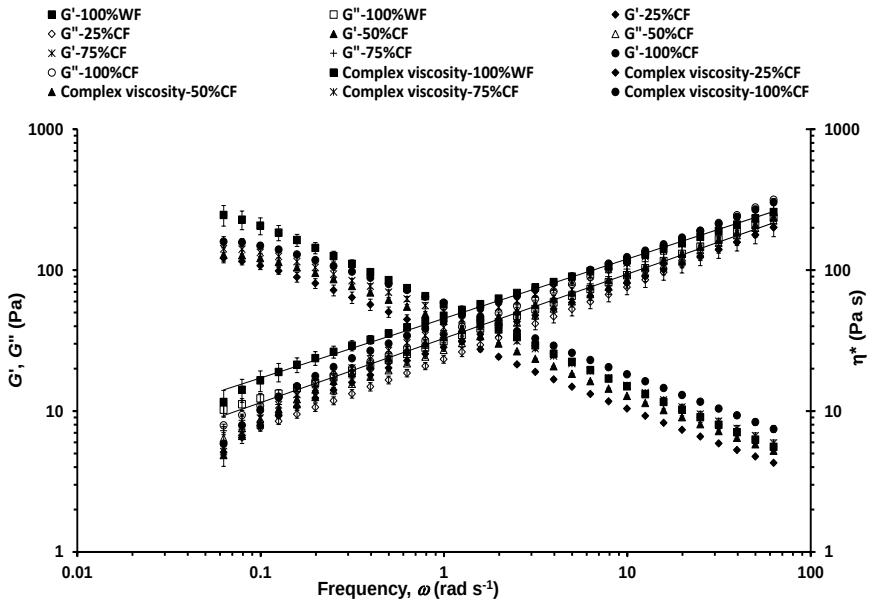

Figure 1. Mechanical spectra data for the muffin control gluten batter and batters with wheat flour (WF) replaced by different percentages of chickpea flour (CF). Mean values of nine measurements ± standard deviation.

In addition, both G' and G'' increased considerably with increasing ω. In all the formulations the frequency dependence of G' and G'' correspond to straight lines in the log–log plot; therefore they can be fitted to the power law equations (Eqs. (2) and (3)):

$$G' = G'_0 \, \omega^{n'} \tag{2}$$

$$G'' = G''_0 \, \omega^{n''} \tag{3}$$

where G'_0 and G''_0 are storage and loss moduli at 1 rad s^{-1}, respectively, and n' and n'' (both dimensionless) denote the ω dependence of the two moduli. According to Campo-Deaño and Tovar [20], the difference ($G'_0 - G''_0$) can be used as a measure of *gel strength*.

According to these parameters the five batters showed frequency dependence specifically in CF-based batters (Table 3). Better fits ($R^2 > 0.99$)

were observed for the power law functions describing the relationship between loss modulus and frequency, indicating that the behavior of G' was slightly less linear than that of G''. The control gluten batter (100%WF) showed the highest G' value throughout the frequency range, which means that more deformation energy can be stored in this sample. This effect is compatible with the increasing viscous component (higher G'') of the batter prepared without gluten (100%CF), which means that more energy can be lost during shear of 100% CF batter. These results are also in accordance with the highest value of *gel strength* (Table 3) and G^* value and lowest tan δ for gluten batter (stress sweep section). However, remarkably, the G' and G'' values of 100%WF and 100% CF throughout the frequency range are not statistically different, as might be expected when observing Figure 2, where the η^* values of the two batters prepared with only one flour type were higher in the 100%WF and 100% CF batters for low and high frequencies, respectively.

On the other hand, reducing WF by increasing the CF levels increased the values of both G' and $G,''$ which is reflected in the increase in the parameters G'_0 and G''_0 (Table 3). The 25%CF batter showed the lowest values of both parameters, followed in increasing order by 50%CF, 75%CF, and 100%CF. These differences observed in the viscoelastic behavior in the different batters can be attributed to the different protein and starch source. In this way, the protein contents provided by the supplier were 10.2 and 19.4% for WF and CF, respectively, while the total carbohydrate content of CF is 55% w/w with a lower starch content reported by Xu et al. [21], ranging between 34.9 and 42.9% w/w. And carbohydrate (85% w/w) and starch (78.8% w/w) contents have been reported for WF [22]. Navickis et al. [23] reported that higher viscoelastic moduli were observed at higher protein levels. Nevertheless, differences with respect to the 100%WF sample were only significant for the three partial replacements (25%CF, 50%CF, 75%CF batters) in the case of G'_0, and for the lower levels of WF replacement (25%CF, 50%CF) in the case of G''_0. Moreover, these differences can also be attributed to the different functional properties of the two types of flours [24].

The corresponding n' and n'' values were significant higher than control gluten and very similar among CF-based batter, showing that all these latter formulations exhibit higher ω dependence associated which it could be associate with a reduction in the structural stability with increasing CF percentage in the batter. Moreover, although differences were not significant, the increase in the G'_0 and G''_0 values with increasing WF replacement. Thus, all these results seem to indicate that all the muffin batters containing CF at any level had a lower structural quality than those of the control gluten batter.

Table 3. Effect of wheat flour replacement on power law parameters of Eqs. (2) and (3) and quality factor of Eq. (4) from frequency sweep tests of the muffin batter at 25°C

Type of formula	G'_0 (Pa $s^{n'}$)	n'	R^2	G''_0 (Pa $s^{n''}$)	n''	R^2	$G'_0 - G''_0$ (Pa s^n)	Q
100%WF	45.36±3.32^{a}	0.4235±0.0242^{b}	0.996±0.003	32.80±1.01^{a,b}	0.4563±0.0122^{b}	0.997±0.002	12.56±2.36^{a}	8.05±0.16^{a}
25%CF	26.99±2.87^{d}	0.5050±0.0295^{a}	0.993±0.003	24.72±3.57^{d}	0.5023±0.0253^{a}	0.999±0.000	2.27±0.88^{b}	6.93±0.20^{b}
50%CF	30.23±0.67^{c,d}	0.5098±0.0096^{a}	0.988±0.009	27.38±0.54^{c,d}	0.5020±0.0091^{a}	0.999±0.001	2.86±0.15^{b}	7.06±0.14^{b}
75%CF	34.04±1.23^{b,c}	0.5220±0.0172^{a}	0.986±0.003	31.46±0.42^{b,c}	0.5139±0.0229^{a}	1.000±0.000	2.58±1.15^{b}	6.93±0.32^{b}
100%CF	39.45±0.68^{a,b}	0.5320±0.0131^{a}	0.987±0.004	36.81±1.07^{a}	0.5355±0.0080^{a}	1.000±0.000	2.64±0.88^{b}	6.68±0.19^{b}

Values are given as mean (n = 9) ± standard deviation. a-h Different letters in the same column mean significant differences ($P < 0.01$) among samples according to LSD multiple range test. WF: wheat flour; CF: chickpea flour; XG: xanthan gum; WP: wheyprotein; INL: inulin; tan δ, loss tangent; G'0, G"0, n', and n," regression coefficients relating G' or G" and frequency (f).

Additionally, the effect of WF replacement was evaluated by means of the quality factor Q (Eq. (4)), a term frequently used in mechanical oscillatory systems. It is a dimensionless parameter that expresses the degree of damping of an oscillator.

$$Q = 2\pi(G'_0/G''_0)\ \omega^{(n'-n'')} \quad (4)$$

The Q factor unifies parameters that provide structural information of different kinds: G'_0 and G''_0 are related to the strength of the intermolecular interactions, and n' and n'' to the extent and stability of the protein structural system [25]. As we can see in Table 3, the control gluten sample showed significantly higher Q values (at angular frequency 10 rad s^{-1}), indicating again a higher developed structure of this batter on short time scales [26].

Temperature Sweep Tests

To understand the structural changes that take place in the various reformulated muffin batters during heating, the linear viscoelastic properties were studied during temperature sweeps from 25 to 125°C, trying to simulate the batter's behavior in the oven. The effect of reducing WF (at 0, 50, and 100% reduction levels) can be seen in Figure 2a, which shows the G' and G'' moduli during heating. Similar heating patterns were observed in the 25%CF and 75%CF batters (data not shown). In turn, Fig. 2b shows the loss tangent, tan δ, as a function of increasing temperature in all the batters prepared with decreasing quantities of WF (25%CF, 50%CF, 75%CF and 100%CF), together with the control gluten batter. As indicated above, at the beginning, the non-isothermal heating tests were carried out in the nonlinear viscoelastic range.

It can be seen that at the beginning of the heating (at 25°C) G'' was higher in magnitude than G' (Figure 2a), reflecting the liquid-like behavior of all the batters, although the initial G', G'', and tan δ values (Figure 2b) varied among batters with and without added CF. Reduction of WF from the level in the control gluten batter (at all the percentages used) decreased the G' and G'' values (at 25°C) and decreased viscoelasticity (values of tan δ became higher), meaning that the presence of only gluten (100%WF) increased the viscoelasticity of the batter system before heating. At 25°C, tan δ was 1.1 for the control gluten sample, 1.5 for 25%CF, 1.6 for both 50%CF and 75%CF, and 1.7 for 100%CF, although without significant differences among the batters containing CF.

In all the batters, the initial increase in temperature produced a decrease in the values of both moduli, associated with a decrease in viscoelasticity

(increasing tan δ values). This decrease might be associated with CO_2 formation in the batter, diffusion into occluded air cells, and expansion, reducing the density of the batter [2]. The initial decrease in the viscoelastic moduli was more noticeable for 50% WF replacement than for 100% (Figure 2a). However, differences in the initial heating pattern between the control gluten and the CF batters can be observed more easily in Figure 2b. The initial increase in temperature increased the values of tan δ, but, while this increase was small and gradual in the control gluten batter, in the CF-based batters an evident change in the shape of the curve was observed. Especially in the 25%CF, 50%CF, and 75%CF batters, the tan δ values increased considerably between 25 and 45°C, which may be associated with the lower connectivity of the batters without gluten, so that the 100%WF structural system was more elastic (lower tan δ) and stronger (higher G') between 25 and 75°C than those of CF-based batters. This lower viscoelasticity reveals a higher complexity of the system structure with CF, once more reflecting a possible thermodynamic incompatibility between polysaccharides, proteins, or both occurring in batters formulated with mixtures of both flours. It was found that many proteins are thermodynamically incompatible in aqueous solutions [27]. During mixing of flour with water, albumins, globulins, water-soluble starch (from damaged starch granules), and pentosans form a liquid aqueous phase. This is immiscible with glutelins and gliadins, which form a separate gluten phase. Chickpea seed protein consists of globulins (56.0%), glutelins (18.1%), albumins (12.0%), prolamin (2.8%), and residual proteins [18].

In contrast, in addition to albumins and globulins, wheat protein mainly consists of gluten (85%) composed of monomeric gliadins and polymeric glutenins [28]. Moreover, total starch content was 53.4% on dry basis for raw CF [29], and 78.8% for WF [22].

With increasing temperature (Figure 2a), the G' and G'' of the all batters increased markedly between 68 and 82°C, and the cross-over of G' and G'' was reached, which was also associated with increased viscoelasticity (decreasing values of tan δ). Gel point, most commonly defined as the time at which G' and G'' intersect [30], generally occurs as a result of the early stage of starch gelatinization [31]; this stage corresponds to when starch granules swell and melt. Finally, the G' and G'' of the samples increased more slowly from 75 to 125°C until a maximum value was reached; this period is associated with complete starch gelatinization and/or protein denaturation or coagulation. Note that the behavior pattern of G' and G'' between the temperature where there was cross-over of the two moduli (T_{gel}) and the final heating temperature was fairly similar in all the batters, with close maximum values of the two moduli.

Throughout the temperature sweep, the increase in temperature also reduced the values of tan δ (values closer to 0) in a similar fashion (Figure 2b).

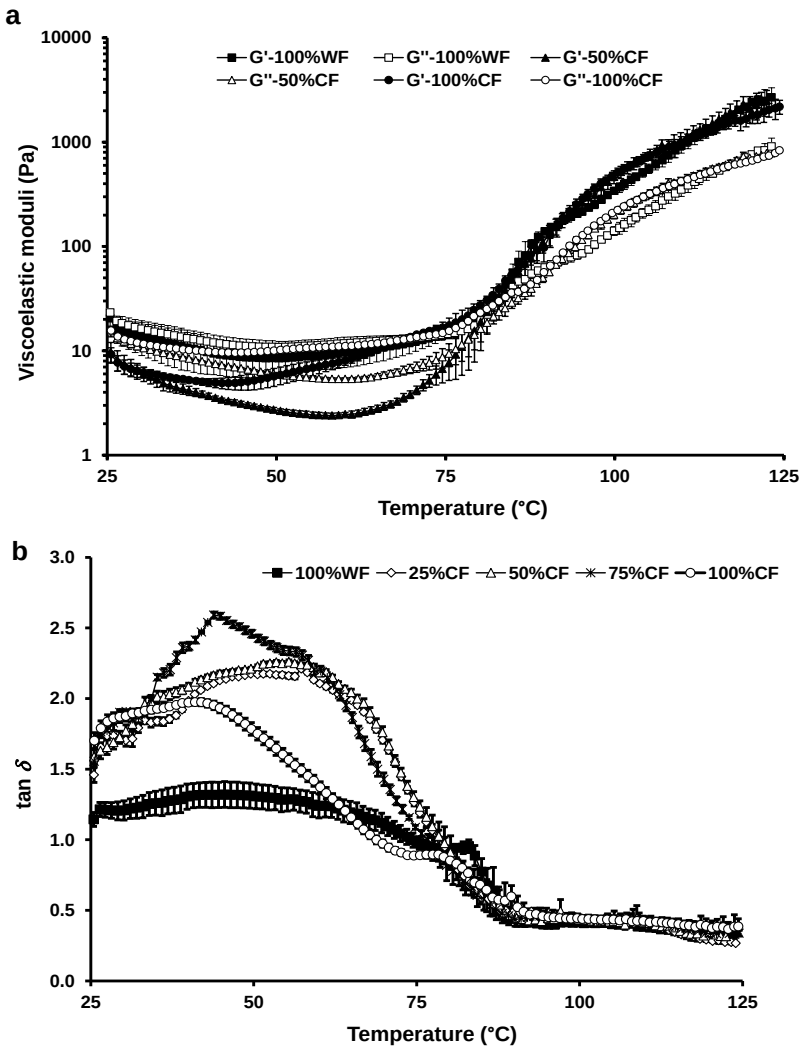

Figure 2. Storage modulus (G') and loss modulus (G'') (a), and loss tangent, tan δ, (b) as a function of increasing temperature in the muffin control batter and batters with wheat flour (WF) replaced by different percentages of chickpea flour (CF). Mean values of nine measurements ± standard deviation.

The effect of replacing WF with increasing quantities of CF on the gel points of the batters during heating is shown in Table 4. WF reduction and replacement with CF significantly ($P < 0.05$) affected the starting gel point time and temperature of the batters. Total absence of gluten (100%CF) resulted in a significant decrease in the gel point in comparison with the 25%CF and 50%CF reduced-WF batters. In addition, the presence of both flour types increased the starch gelatinization temperature of the batter. This delay in the temperature and time of the inflexion point in the samples containing either WF or CF could be crucial to avoid earlier thermosetting and give enough time for appropriate air and vapor expansion during baking [2]. However, although 100%CF batter had the lowest starting gel point time and temperature, there were non-significant differences between the gel point values of this gluten-free muffin batter and the 100%WF batter.

It can be seen that at the beginning of heating the complex viscosity (η^*), defined by $G^*/i\omega$, was significantly higher in magnitude in the 100%WF sample (Table 4), again reflecting the more solid-like behavior of gluten batter at 25°C. As shown, from 75°C upwards the temperature sweep profiles were very similar (Figure 2a), a priori suggesting a very similar response and rheological behavior of all the batters during baking. Nevertheless, after complete starch gelatinization there were still some significant differences in the values of the rheological properties of the batters. The η^* at the final temperature of 125°C was significantly lower in the batters prepared with 75 and 100% WF reduction compared with the batter with 25% WF reduction (Table 4). However, these η^* values at 125°C should be judged with caution, as sample desiccation was clearly observed occasionally at this temperature.

The non-isothermal kinetic relation based on the experimental data and regression analysis was determined following the steps described by Rhim et al. [32] for a linearly increasing temperature system. In this study, the temperature range selected for the kinetic analysis was the range from the starting gel point temperature (cross-over of G' and G'') up to 100°C, which was therefore considered as the temperature where G' achieved its maximum value. Data from 100 to 125°C were discarded because of their high dispersion, which is related to sample desiccation occurring at higher temperatures.

Table 4. Effect of wheat flour replacement on gel points, complex viscosity values, and kinetic parameters from Eqs. (5) and (6) during temperature sweeps of the muffin batter

	Gel point (G' – G'' cross-over)		Onset and endset complex viscosities		Non-isothermal kinetic parameters						
Type of formula	Time (t_{gel})(s)	Temperature (T_{gel}) (°C)	η^* at 25°C (Pa s)	η^* at 125°C (Pa s)	Order (n)	$\ln k_0$ (k_0 in $Pa^{(1-n)}\ s^{-1}$) ($n = 0$)	E_a (kJ mol^{-1}) ($n = 0$)	R^2 (Eq. 5) ($n = 0$)	MSE (Eq. 5) ($n = 0$)	R^2 (Eq. 5) ($n = 1$)	MSE (Eq. 5) ($n = 1$)
100%WF	1890.7±185.3^{a,b}	74.5±4.9^{a,b}	49.4±5.9^{a}	4785.6±309.4^{a,b}	0.19	36.53	113.43	0.995	0.021	0.950	0.056
25%CF	2064.9±76.0^{a}	79.0±2.0^{a}	30.4±5.1^{b}	5929.5±655.3^{a}	0.94	47.12	144.68	0.992	0.042	0.999	0.015
50%CF	2076.9±109.3^{a}	79.4±2.9^{a}	29.1±4.3^{b}	4856.9±580.9^{a,b}	0.71	44.25	135.63	0.992	0.045	0.997	0.027
75%CF	2025.2±84.6^{a,b}	78.0±2.3^{a,b}	36.4±3.4^{b}	4130.9±459.2^{b}	0.70	47.38	145.33	0.993	0.041	0.995	0.031
100%CF	1701.2±54.7^{b}	69.5±1.4^{b}	28.9±2.1^{b}	3873.5±438.2^{b}	-1.73	66.88	204.38	0.954	0.175	0.946	0.220

Values are given as mean ($n = 9$) ± standard deviation. ^{a,b} Different letters in the same column mean significant differences ($P < 0.05$) among samples according to Bonferroni multiple range test. WF: wheat flour; CF: chickpea flour. t_{gel}: starting gel point time; T_{gel}: starting gel point temperature; η^*: complex viscosity; n: reaction order; k_0: frequency factor; E_a: activation energy. MSE: mean square error.

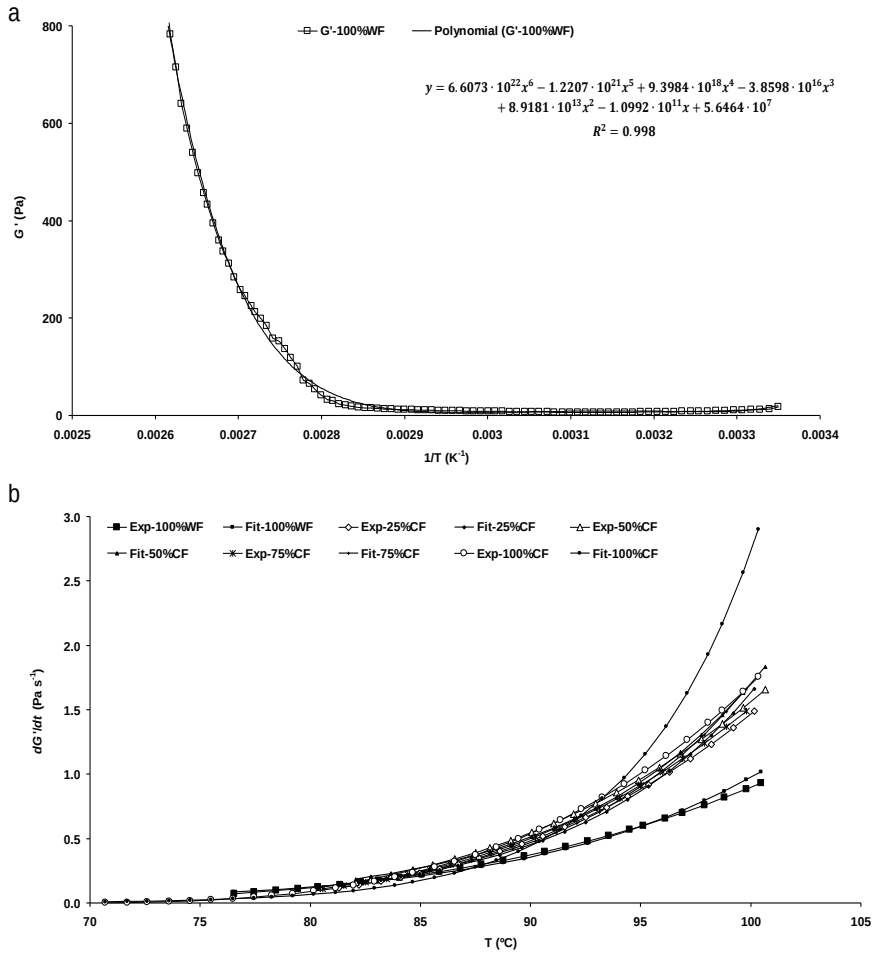

Figure 3. (a) Sixth-order polynomial fit approximating the change of elastic modulus (*G'*) vs. inverse of absolute temperature between 25 and 100°C during non-isothermal heating of muffin batter made only with wheat flour. (b) Applicability of zero-order kinetics for muffin batters during thermal gelatinization.

The kinetic equation can be converted to Eq. (5) in terms of rheological parameters (*G'* and *dG'*), as described by Ahmed et al. [31], Alvarez et al. [33], Yoon et al. [34]. The negative sign of kinetic equation is substituted by a positive sign because of the increase *G'* during heating (positive *dG'*).

$$\ln\left(\frac{1}{G'^{n}}\frac{dG'}{dt}\right) = \ln k_0 - \left(\frac{E_a}{R}\right)\left(\frac{1}{T}\right) \quad (5)$$

where G' (Pa) and dG'/dt (Pa s^{-1}) denote the elastic modulus and the derivative of G' with respect to time t, respectively, k_0 the pre-exponential or frequency factor ($Pa^{(1-n)}$ s^{-1}), n the reaction order, E_a the activation energy (J mol^{-1}), T the absolute temperature (K), and R the universal gas constant (8.314 J mol^{-1} K^{-1}).

Derivatives of experimental data are usually calculated by the following methods: (1) graphical differentiation, (2) polynomial curve fitting and differentiation of the fitted equation, and (3) numerical differentiation [32].

In this study, derivatives of experimental data were calculated by sixth-order polynomial fits approximating the change of G' vs. t (for all the cases, the corresponding mean R^2 value was 0.999 ± 0.002) in the complete temperature range considered (from 25 to 100°C). As an example, Figure 3a shows the sixth-order polynomial fit approximating the change of elastic modulus vs. the inverse of the absolute temperature during non-isothermal heating of 100%WF batter.

A multiple linear regression was then used with the muffin batter kinetic data set to determine the order of the reaction (n) after changing the above equation into the following linear form (Eq. (6)):

$$\ln\left(\frac{dG'}{dt}\right) = \ln k_0 + n\ln G' - \left(\frac{E_a}{R}\right)\left(\frac{1}{T}\right) \quad (6)$$

The reaction orders obtained from the above equation for muffin batters are shown in Table 4. The values of the kinetic parameters were derived from the mean curves obtained for each batter. For this reason, standard deviations and mean comparisons from statistical analysis are not given for these parameters. The control gluten sample (100%WF) showed a reaction order very close to zero, whereas the calculated n values were closer to one for the muffin batters made with mixtures of both wheat and chickpea flour. Unexpectedly, the n of the above Eq. (6) was found to be negative for the 100%CF batter. However, the reaction order was further verified by considering n = 0, and n = 1, in Eq. (5), which confirmed a better fit for zero-order reaction kinetics in the batters made without mixtures (100%WF and 100%CF), as well as a better fit for first-order reactions in the case of the batters containing both types of flour (Table 4). However, note that for the zero-order reactions the R^2 values were higher than 0.990, except for the

100%CF batter (0.954). Then, although by considering $n = 1$ the R^2 of the 25, 50, and 75%CF batters increased slightly compared with zero-order reactions, the applicability of first-order reaction kinetics for 100%WF batter meaningfully decreased the goodness of the fit. Consequently, zero-order reaction kinetics was considered in all five batters in order to compare the values of the kinetic parameters obtained (Figure 3b). Zero-order reaction kinetics has also been reported for CF slurries in a wide moisture range from 1:5 to 1:2 flour-to-water ratio [33].

There was an increase in both the $\ln k_0$ and the E_a values of the muffin batters with the increase in the percentage of WF replacement. The magnitude of E_a was 113.4 and 204.4 kJ mol^{-1} for the 100%WF and 100%CF batters, respectively. The lower E_a in control gluten batter implies that it was more favorable for gelatinization. In accordance with Matos et al. [4], other ingredients such as sugar, egg white powder or egg white liquid, milk, baking powder, salt, vegetable oil, hydrocolloids and emulsifiers could be incorporated in reduced-WF formulations to improve the final product quality. However, according to Ahmed et al. [31] the significant increase in E_a values for gelatinization of 100%CF batter might also be attributable to the fact that the starch granules cannot swell to their equilibrium volume because of limited availability of water. Hydrocolloids are used as gelling agents in puddings, jellies, and aspics, as starch retrogradation inhibitors in breads and batters, and as water-binding agents in gluten-free foods [35].

Creep and Recovery Tests

This transient test produces creep and recovery compliance data, *J(t)*, over longer time scales than SAOS measurements [16]. So these experiments can cause irreversible breakdown of short-range interactions; thus providing information about the relative long-range properties of physical systems [12].

Moreover, the *J(t)* values from the creep curves can be used to calculate the relaxation modulus *G(t)* [36], which can be fitted with time to the power law using Eq. (7).

$$G(t) = St^{-n} \tag{7}$$

where S (kPa s^n) is the gel strength and n is the relaxation exponent, both characteristic parameters of each kind of structure [26, 37].

Figure 4 shows creep-recovery compliances for control gluten batter (100%WF) and batters with WF replaced by 50 and 100% of CF (50%CF and 100%CF, respectively). There are clearly two trends, 100%WF batter showed

the lowest *J(t)* values during creep stage followed by 100%CF batter which indicates that both batters were more dense and rigid than the rest of the CF-based batters. Moreover, 100%WF batter also showed the lowest *J(t)* values during recovery stage meaning that it is the most time-stable batter. In contrast, 50% WF batter showed the highest *J(t)* values during creep and recovery stages indicating that during loading there may been a rupturing of bonds meaning that the batter structure was more deformed, and therefore, more flexible, and these bonds were irreversibly broken. The creep-recovery curves of 25%CF and 75% CF batters were quite similar to that of 50%CF (data not shown).

These experimental *J(t)* data from creep-recovery curves of all the batters were consistent with the parameters found in Table 5. Specifically, control gluten batter and 100%CF batter had the higher values of gel strength (*S*) than the rest of the CF-based batters (25%CF, 50%CF and 75%CF). Nevertheless, 100% WF showed the lowest value of relaxation exponent (*n*) while 100%CF showed the highest value and the rest of CF-based batters showed intermediate values. Thus, the presence of gluten seems to produce dense systems with a higher connectivity degree than the CF-based batters. However, the replacement of WF by CF produced also denser systems but less-time stable especially at 100% replacement level. These results are also consistent with the higher values of G^*_{max}, the lower values of tan δ (stress sweep section) and with the lower influence of frequency on both G' and G'' from mechanical spectra found in the control gluten batter respect to those of CF-based batters, discussed earlier. Thus, replacement of WF by CF produced batters with lower structural properties.

Table 5. Effect of wheat flour replacement on power law parameters of Eq. (7) from creep and recovery tests of the muffin batter at 25°C

Type of formula	*S* (kPa s^n)	*n*	R^2
100%WF	$0.054\pm0.003^{a,b}$	0.510 ± 0.026^{c}	0.999±0.000
25%CF	0.041 ± 0.004^{c}	0.597 ± 0.011^{b}	0.999±0.000
50%CF	0.040 ± 0.004^{c}	0.646 ± 0.013^{a}	0.999±0.000
75%CF	$0.047\pm0.002^{b,c}$	0.656 ± 0.005^{a}	0.999±0.000
100%CF	0.063 ± 0.005^{a}	0.656 ± 0.020^{a}	0.999±0.000

Values are given as mean (n = 9) ± standard deviation. [a-d] Different letters in the same column mean significant differences ($p < 0.05$) among samples according to Bonferroni multiple range test. WF: wheat flour; CF: chickpea flour; *S*: gel strength; *n*: relaxation exponent.

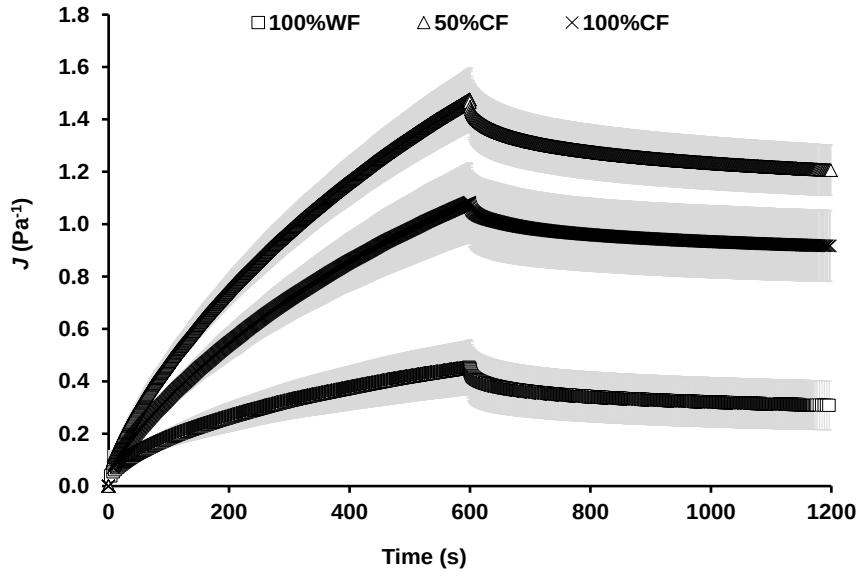

Figure 4. Creep and recovery compliance *J(t)* data for the muffin control batter and batters with wheat flour (WF) replaced by different percentages of chickpea flour (CF). Mean values of nine measurements ± standard deviation.

Steady-State Shear Measurements

Hysteresis Area

Structural systems break down in the accelerating shear rate portion of a rheogram and restructure in the decelerating portion. When a sample is sheared at increasing and then at decreasing shear rates, the observation of the hysteresis area between the curves representing shear stress values indicates that the sample's flow is time-dependent [38, 39, 13]. According to the authors just cited, the area encircled between the ascending and the descending curves is an index of the energy per unit time and unit volume needed to eliminate the influence of time on flow behavior. All the batters showed observable hysteresis (Figure 5). Therefore the presence of hysteresis loops in Figure 5a indicates thixotropic breakdown and behavior of all the muffin batters studied. However, differences in the magnitude of the hysteresis loop were observed among the batters (Table 6). The initial or first hysteresis loop area decreased

as the level of WF replacement in the batter increased. As a result, thixotropy became significantly smaller for the higher WF replacement level (100%CF). Assuming that a hysteresis loop area is an index of the energy needed to destroy the structure responsible for flow time dependence, the experimental data showed 'that control gluten batter was the one that needed the highest energy to break down the structure. On the other hand, 100%WF batter showing the largest loop area is also the one showing the lowest resistance to flow and the strongest structural destruction under identical shearing conditions (Figure 5a). On the other hand, while assays performed within the linear range are non-destructive, elastic or viscoelastic materials tested outside this range may suffer irreversible structural changes [40]. In real production processes viscoelastic fluids are subjected, most of the time, to high shear deformation conditions which are linked to the nonlinear viscoelastic behavior of the material. According to the authors just cited, any rheological material testing should therefore explore how samples behave inside and outside the range of linear viscoelasticity.

Flow Behavior

On the basis of the above results, before analyzing flow behavior a previous shearing of 5 min at 300 s^{-1} was applied to all the batters in order to eliminate or reduce as much as possible the flow time dependence of the batter [39, 41]. On recording shear stress variation with shear rate (forward and backward measurements) in order to guarantee the steady-state shear condition (~10 min), yet again all the samples showed observable hysteresis (Figure 5b). However, for the same shearing conditions the percentage of eliminated hysteresis loop was almost 94% in the control gluten batter (Table 6), and it decreased as a result of increasing the level of WF replacement, being only 35% in the 75%CF batter. When a material is sheared at a constant shear rate, the viscosity of a thixotropic material will decrease over a period of time, implying a progressive breakdown of structure [38]. Therefore, this result reflects a significantly higher destruction by shearing of the internal structure, and thus a faster breakdown rate of batter associations, in the 100%WF sample in comparison with the 100%CF one. It is also interesting to note that after shearing the 100%CF sample the hysteresis loop area of the batter was still higher than the first one obtained without previous shearing. Thus, it seems that the replacement of WF (gluten) by CF make more compacted and entangled structures.

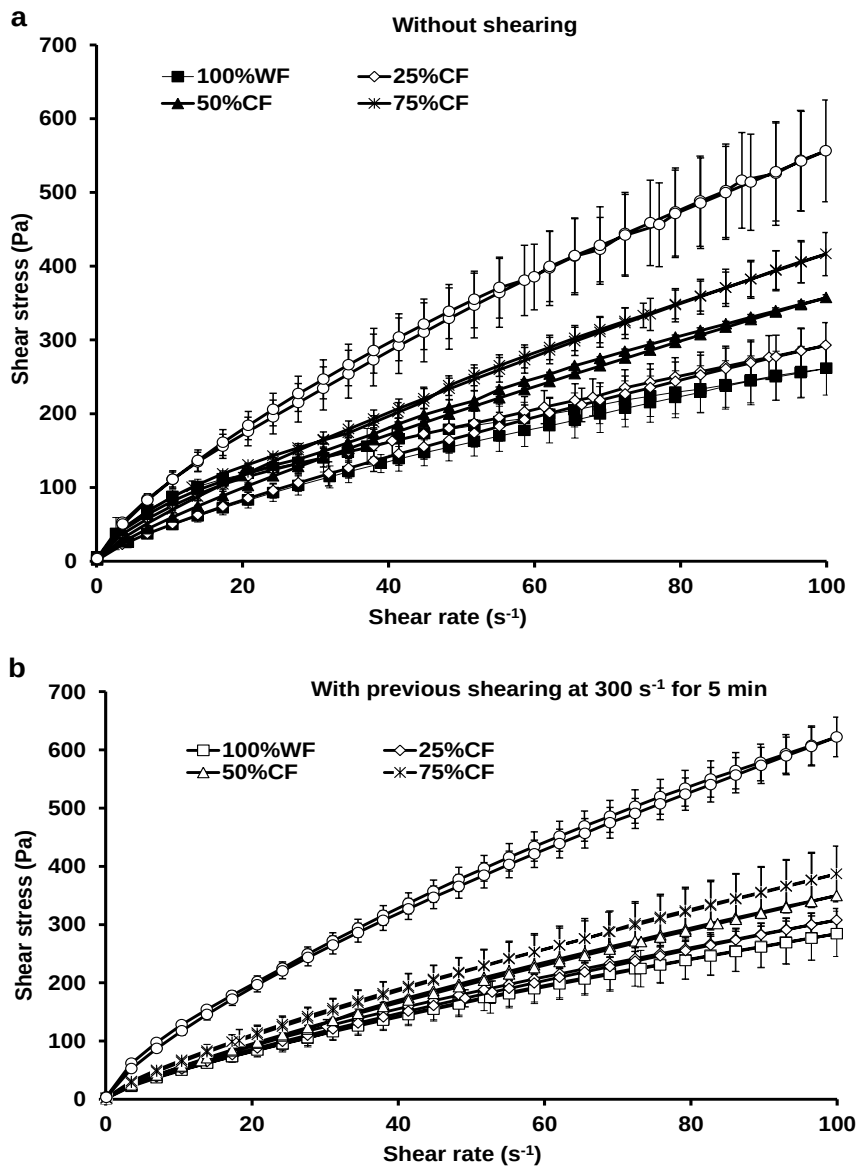

Figure 5. Flow curves measured by increasing (forward measurements) and decreasing shear rate (backward measurements) for the muffin control batter and batters with wheat flour (WF) replaced by different percentages of chickpea flour (CF) without shearing (a), and after shearing the batters at 300 s^{-1} for 5 min (b).

In addition, after reducing the flow time dependence by shearing, sample flow was measured, and all samples showed non-Newtonian shear-thinning behavior with an apparent initial resistance to flow. Assuming a non-linear relationship between shear stress and shear rate, the data obtained were fitted to the Casson (Eq. 8) and Herschel–Bulkley models (Eq. 9) (Rao, 1999) using Bohlin CVO 120 software (v. 06.40):

$$\sigma^{0.5} = K_0 + K\dot{\gamma}^{0.5} \tag{8}$$

$$\sigma = \sigma_0 + K\dot{\gamma}^{n} \tag{9}$$

In Eq. (8), the Casson yield stress (Pa) is calculated as the square of the intercept, $\sigma_0 = K_0^2$, and the Casson viscosity (Pa s) is $\eta_C = K^2$. In Eq. (9), K is the consistency index (Pa s^n), n is the flow behavior index (dimensionless), and σ_0 is the yield stress (Pa). The σ_0 value used in the Herschel–Bulkley model was that obtained by fitting the experimental data to the Casson model, as described by Tárrega et al. [13]. For all models, σ is the shear stress (Pa) and is the shear rate (s^{-1}). As thixotropy had been reduced to a certain extent, models for time-dependent flow behavior were not consideres in this study.

Experimental data fitted well to the Casson model (Table 6), with R^2 values ranging between 0.997 and 0.999. The yield stress values were high – higher than 4 Pa at 100%WF and higher than 12 Pa at 100%CF – indicating plastic behavior. The high yield stress value obtained at the highest replacement level shows that the 100%CF sample had a significantly higher initial resistance to flow, as well as a significantly higher Casson plastic viscosity, suggesting that it is the most dense and entangled structure than all the batters containing different levels of WF (gluten). Experimental data fitted better to the Herschel–Bulkley model, with R^2 values ranging between 0.999 and 1.000 (Table 6). At 25°C, n was significantly lower and K was much higher in 100%CF than in the other batters, indicating greater pseudoplasticity and consistency of the gluten-free under steady-state shear.

Table 6. Effect of wheat flour replacement on hysteresis areas and rheological parameters of Eqs. (8) and (9) describing flow curves of the muffin batter at 25°C

Type of formula	Hysteresis area before shearing (Pa s^{-1})	Hysteresis area after shearing (Pa s^{-1})	Casson model			Herschel–Bulkley model		
			σ_0 (Pa)	η_C (Pa s)	R^2	K (Pa s^n)	n	R^2
100%WF	1974.3±353.1[a]	115.8±47.2[b]	4.23±0.47[b]	2.31±0.32[c]	0.998±0.000	8.60±0.64[b]	0.760±0.013[b,c]	1.000±0.000
25%CF	1438.5±65.9[b]	191.9±73.7[b]	4.49±1.56[b]	2.49±0.12[b,c]	0.998±0.000	8.56±1.76[b]	0.780±0.031[a,b]	0.999±0.001
50%CF	1034.4±281.8[b]	202.0±118.6[b]	3.22±0.29[b]	2.98±0.06[b,c]	0.999±0.000	8.65±0.26[b]	0.804±0.006[a]	1.000±0.000
75%CF	504.1±145.7[c]	297.5±105.8[a,b]	4.89±0.69[b]	3.18±0.40[b]	0.999±0.000	10.02±1.34[b]	0.793±0.023[a,b]	1.000±0.000
100%CF	357.0±71.1[c]	428.8±44.9[a]	12.57±0.81[a]	4.41±0.54[a]	0.997±0.000	19.91±2.00[a]	0.729±0.005[c]	0.999±0.000

Values are given as mean (n = 9) ± standard deviation. [a-d] Different letters in the same column mean significant differences ($P < 0.05$) among samples according to Bonferroni multiple range test. WF: wheat flour; CF: chickpea flour. σ_0, yield stress; η_C, Casson viscosity; n, flow behavior index; K, consistency index.

Batter viscosity is an important physical property as it is closely related to the final quality of an aerated baked product. Martínez-Cervera et al. [42] reported that the increasing viscosity as CF levels rose could be an unfavorable factor for the quality of the final baked product. These negative effects of replacing WF (gluten) with CF could be because of the higher protein, and/or lower starch contents in CF, corroborating previous findings [43].

Three-Step Shear Rate Test

For each batter, Table 7 shows the original and final apparent viscosities, the time taken from the onset of the third stage (after structural breakdown) to achieve rebuild of the viscosity to 90% of the original value, and the recovery percentage of the original viscosity at the end of the test, i.e., after a rebuild time of 600 s. Original apparent viscosity values at 0.1 s^{-1} (low shear rate) were highest for the control gluten batter (100%WF), agreeing with its higher viscoelasticity, whereas η_o values were significantly lower in all three batters containing both flour types, without significant differences between them. In turn, the 100%CF batter had an intermediate η_o value. Similar results were obtained for storage moduli (elasticity) of the batters from frequency sweeps (Table 3).

When the shear ceases, the internal structure is recovered when the batter is left to rest for a more or less prolonged (recovery) period time. The apparent viscosity value after restructuring for 600 s after cessation of flow (η_f) was significantly ($P < 0.05$) higher for the 100%CF batter (Table 7). As a result, the percentage of viscosity recovery calculated with respect to the η_f value was 83.6% for 100%WF, whereas the values obtained ranged between 106.8 and 146.0 for batters containing increasing percentages of CF. This percentage increased significantly as the level of WF replacement increased, being 146.0% for the full amount of replacement, i.e., for the 100%CF sample. With regard to the time required to recover 90% of the original apparent viscosity after structure breakdown (Table 7), it could not be obtained for the control gluten batter, being higher than the rebuild time considered (>600 s). However, this time decreased significantly with increasing percentage of WF replacement, and the lowest value corresponded to the maximum level of WF replacement (100%CF sample).

A low viscosity recovery percentage and a long rebuild time indicate that the 100%WF sample is more thixotropic than the rest of the batters with higher recovery percentage and shorter rebuild time. Therefore, this test evidences and confirms that control gluten batter made only with gluten had the most thixotropic behavior, whereas for all the batters containing CF protein

the thixotropy decreased significantly as the gluten content decreased to zero. In contrast, under SAOS measurements 100%WF batter had higher viscoelasticity (Table 3) and also more long-lasting memory, exhibiting more elasticity (Table 5) in addition to viscous behavior.

Table 7. Effect of wheat flour replacement on viscometry rebuild analysis from three-step shear rate tests at 25°C of the muffin batter

Type of formula	η_o (Pa s)	η_f (Pa s)	*Time to recover 90% of* η_o (s)	*Percentage of viscosity recovery* (%)
100%WF	51.7±1.6^{a}	43.2±0.1^{b,c}	>600	83.6±2.4^{d}
25%CF	38.2±2.4^{c}	40.7±1.5^{b,c}	121±25^{a}	106.8±6.5^{c}
50%CF	34.5±1.6^{c}	40.2±1.1^{c}	52±3^{b}	111.9±3.3^{b,c}
75%CF	35.3±0.2^{c}	43.6±1.2^{b}	26±11^{b,c}	123.5±3.3^{b}
100%CF	45.4±0.9^{b}	66.3±0.7^{a}	10±1^{c}	146.0±4.0^{a}

Values are given as mean (n = 9) ± standard deviation. $^{a-d}$ Different letters in the same column mean significant differences (P < 0.05) among samples according to Bonferroni multiple range test. WF: wheat flour; CF: chickpea flour. η_o, original apparent viscosity at the onset of the first stage; η_f, final apparent viscosity at 660 s (after 600 s of recovery).

It is also interesting to observe that all four batters containing CF showed a different response to flow in comparison with the control gluten; their percentages of viscosity recovery were much higher than 100, reflecting that the viscosity of the CF batters after shearing and restructuring was even higher than that of their resting structures. All four CF formulae exhibited a short, rapid build-up of viscosity to a highly structured state after shearing. In the case of the 100%CF sample, 90% of the internal structure was recovered when the batter was left to rest for a very short time (10 s); Newtonian fluids recover almost instantaneously upon removal of shear, but the recovery time of non-Newtonian fluids depends on both particle orientation and collision effects during structure rebuilding. It is obvious that the 100%CF sample exhibited the fastest rate of structure rebuilding. The results show that the reduction or total absence of gluten in the batter, i.e., of gluten, significantly accelerates the recovery rate when the viscosity function is considered alone, without taking the elastic component into account.

Applicability of the Cox–Merz Rule

Another of the goals of this study was to search for relationships between oscillatory viscoelastic properties and steady-state rheological properties. The Cox–Merz rule states that the complex viscosity (η^*) at a specific oscillatory frequency (ω) is equal to the apparent viscosity ($\eta_{app} = \sigma/\dot{\gamma}$) at a specific shear rate ($\dot{\gamma}$), when $\omega = \dot{\gamma}$ (Eq. (10); [44]). When this rule is valid, the rheological food properties can be obtained by either oscillatory or steady-state shear experiments, which is particularly useful because of the characteristics and limitations of each kind of experiment findings [41].

$$\eta^*(\omega) = \eta_{app}(\dot{\gamma}) \mid_{\omega=\dot{\gamma}} \tag{10}$$

As Figure 6 shows, the Cox–Merz rule could not be used directly. As commonly observed in food products, for all the batters the η^* magnitudes were always higher than the η_{app} magnitudes. This is why oscillatory shear produces a small deformation which does not completely disturb the microstructure of the batter sample, whereas steady shear produces a large deformation, which induces structural changes findings [45]. On the other hand, for each batter it is possible to see that the two lines are not parallel to each other, suggesting that a linear relationship between viscosities cannot be applied. Therefore a power (Eq. (11)) modified Cox–Merz rule was used for evaluation of the rheological properties.

$$\eta^*(\omega) = \alpha[\eta_{app}(\dot{\gamma})]^{\beta} \mid_{\omega=\dot{\gamma}} \tag{11}$$

Table 8. Effect of wheat flour replacement on power model coefficients from modified Cox–Merz rule of the muffin batters at 25°C

	Non-linear regression		
Type of formula	Multiplicative constant, α	Power index, β	R^2
100%WF	1.297	1.189	0.998
25%CF	0.783	1.337	0.997
50%CF	0.931	1.305	0.998
75%CF	0.843	1.482	0.967
100%CF	0.827	1.241	0.980

Values are given as mean (n = 9) ± standard deviation. WF: wheat flour; CF: chickpea flour.

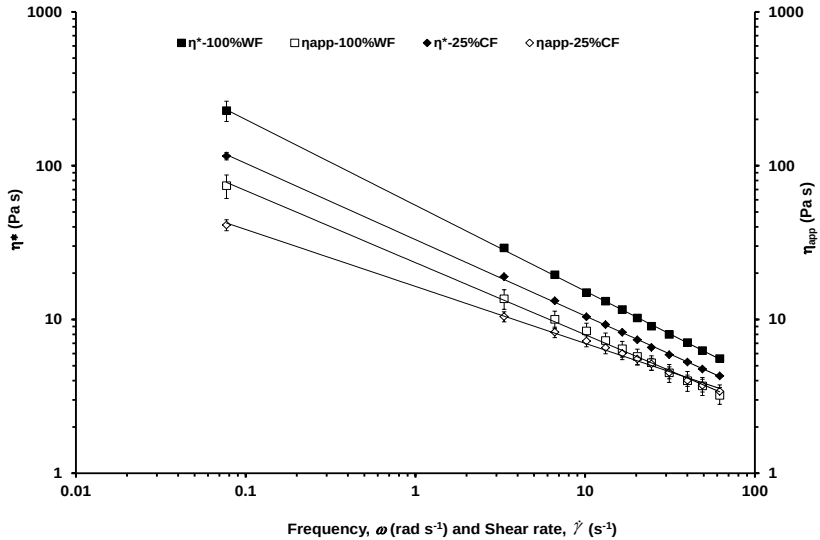

Figure 6. Applicability of Cox–Merz rule for the control gluten batter and the batter in which wheat flour (WF) was replaced by chickpea flour (CF) at the lowest replacement level (25%CF).

The values of parameters α and β (Eq. (11)) are shown in Table 8, with R^2 higher than 0.99 for the 100%WF, 25%CF, and 50%CF batters. The difference between viscosities was highest for the control gluten batter (highest value of α). The power indices ranged from 1.189 for 100%WF to 1.482 for 75%CF, indicating that the relationship between the viscosities was clearly non-linear (β values are not close to 1).

Color and Quality Parameters of Muffin Batter

An increase in WF substitution produced a decrease in the lightness of the batter and an increase in the redness and yellowness values (Table 9), that is to say, the batter acquired a more saturated yellowish color. The a^* and b^* values were significantly ($P < 0.05$) higher in the formulations with a higher degree of WF substitution with CF. As a result, it is concluded that the reduced-WF muffin batters had a more orangey color than the control gluten batter (100%WF). The ΔE^* values were in excess of 3 units for all the batters when compared with this control, therefore these differences are clearly appreciated by the human eye.

Table 9. Effect of wheat flour replacement on color parameters and other physical properties of the muffin batters at 25°C

Type of formula	Color parameters				Other physical properties			
	*L**	*a**	*b**	ΔE*	SG (g l^{-1})	TSS [g 100 g^{-1} (w/w)]	pH	Water content (%)
100%WF	66.85±0.09[a]	1.52±0.06[d]	14.47±0.06[e]	0.00	0.97±0.00[b]	21.8±0.24[c]	7.82±0.02[b]	13.28±0.80[b]
25%CF	66.55±0.20[b]	2.04±0.25[c]	19.25±0.34[d]	4.83±0.34[d]	0.97±0.01[b]	21.0±0.25[c]	7.67±0.01[c]	16.19±0.77[a]
50%CF	62.67±0.16[d]	2.77±0.08[b]	23.08±0.27[c]	9.66±0.20[c]	1.01±0.01[a]	25.0±0.21[b]	7.48±0.02[d]	14.80±0.80[a,b]
75%CF	63.24±0.23[c]	2.87±0.15[b]	24.20±0.56[b]	10.47±0.51[b]	0.98±0.01[b]	24.2±0.34[b]	7.49±0.02[d]	17.43±0.62[a]
100%CF	62.48±0.18[d]	3.26±0.23[a]	25.33±0.27[a]	11.84±0.31[a]	0.94±0.01[c]	26.7±0.54[a]	7.90±0.01[a]	13.38±0.83[b]

Values are given as mean (n = 9) ± standard deviation. WF: wheat flour; CF: chickpea flour. ΔE*, total color difference; SG, specific gravity; TSS, total soluble solids.

The effect on specific gravity of WF replacement with CF can also be seen in Table 9. For the replacement levels of 25% and 75%, the SG values were not significantly different from those of the control gluten batter. A significant increase in SG was found for the 50%CF sample, whereas a significant decrease in SG was observed for the 100% WF replacement. SG has also been found to decrease in muffin batter on replacing sucrose with sucralose: polydextrose mixtures [42].

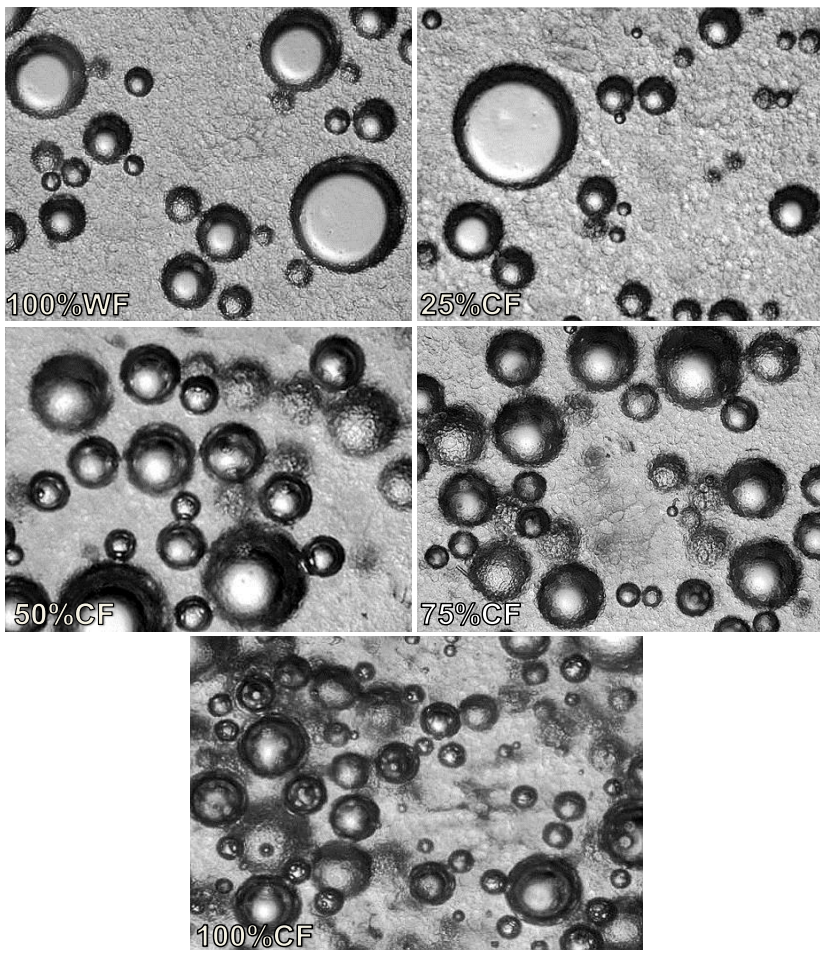

Figure 7. Optical microscopy images for the control gluten batter (100%WF) and batters prepared with increasing quantities of chickpea flour (CF) replacing wheat flour (WF) (25%CF, 50%CF, 75%CF, and 100%CF) (×10).

Lower SG is associated with higher aeration of the batter [46], indicating a higher capacity to incorporate air bubbles during beating and retain them [47]. However, according to Martínez-Cervera et al. [2], the SG values do not provide information about bubble size or distribution. The TSS content of the muffin batter increased significantly as a result of increasing the percentage of WF replacement which could be ascribed to higher protein content. Moreover, 100%CF batter also had the highest pH (Table 9). In comparison with the 100%WF and 100%CF samples, batters made with 25, 50, and 75% WF reduction levels showed significantly ($P < 0.05$) lower pH and higher water mositure. Higher moisture can be associated with higher water absorption due to the presence in these batters of two different sources of protein and starch. However, there were no significant differences in the moisture determined in the batters prepared with the 0 and 100% WF reduction levels.

Microscopy Images of Muffin Batter

Micrographs obtained for the control batter, and for the 25, 50, 75, and 100% WF-replaced batters are shown in Figure 7. When WF was replaced by increasing quantities of CF, a change in air bubble size and quantity was observed: the number of larger-diameter air bubbles tended to decrease, and generally the number of air bubbles present in the batters seemed to increase. In 50%CF and 75%CF batters, an intermediate size of air bubbles was found. As the percentage of WF substitution increased until 100%, an increase in the amount of air in the 100%CF batter was observed, in agreement with its lower SG value. This reduction in bubble size could be the consequence of the lower elasticity of the WF-reduced batters, favoring bubble buoyancy [2]. As the beating energy provided to all the batter formulations was the same, a lower elasticity may have allowed the larger air cells to coalesce and escape while retaining the small ones. Nevertheless, during baking, a second step takes place: the air cells are expanded by CO_2 and the vapor pressure generated, resulting in the formation of the final gas cells, which influence the texture of the finished product [6]. In principle, a larger number of small gas nuclei in the batter is a positive factor for final quality, as it will favor the formation of tiny air cells that can enlarge during baking, which in turn favors height and volume gain [2]. One explanation for the greater number of air bubbles found in the gluten-free muffins is that the early starch gelatinization advanced batter thermosetting, which favoured bubble retention. More research is needed to

get an understanding of the influence of this higher number of small air bubbles in the batter on the final quality of the baked muffin.

CONCLUSION

SAOS measurements indicated that muffin batters made with CF at any level are more deformable, with a lower degree of conformational stability than the 100%WF control gluten batter. Moreover, 100%WF batter could be characterized as a weak gel, while batters with partial and total WF replacement presented a weaker structure. The process of muffin batter gelatinization under non-isothermal conditions was well described by first-order reaction kinetics. The lower activation energy (113.43 kJ mol^{-1}) of control gluten batter implies that it was more favorable for gelatinization. Creep and recovery tests showed that the 100% WF and 100%CF batters showed the lowest compliances values during creep stage meaning that both batters were dense systems. Nevertheless, control gluten batter was more time-stable batter than all CF-based batters as showed its compliances values during recovery stage, and also this gluten batter showed a higher connectivity degree than the all CF-based batters. Under viscous shear flow, 100%WF batter was the most thixotropic, with the lowest viscosity recovery percentage and resistance to flow, the longest rebuild time, and the highest fluidity. Shearing reduced 94% of the flow time dependence in the 100%WF batter, whereas in the 100%CF sample the hysteresis loop increased. In addition, all four CF formulae exhibited a short, rapid build-up of viscosity to a highly structured state after shearing. Flow data fitted very well to the Herschel–Bulkley model, which showed that gluten-free batter had the highest yield stress, pseudoplasticity, and viscosity. Power type modification of the Cox–Merz rule can be successfully used for all the muffin batters studied. SG values would appear to indicate a higher incorporation of air into the 100%CF batter. Future studies will be needed to determine the sensory quality of the baked muffins made with these batters, linking the rheological properties of these batters with the functional properties of proteins and the technological characteristics of the end products.

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BIOGRAPHICAL SKETCH

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Rheology and texture of foods (REOTEXTAL)

PERSONAL INFORMATION

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RESEARCHER

Objectives:

- Rheological, structural and sensorial food characterization.
- Achieve foods with an optimum, stable and standard texture and quality.
- Use and revaluation of undervalued species and fishing industry byproducts for the development of restructured products "on demand" according to consumer preferences with possible addition of non-seafood ingredients and additives.
- Addition of functional ingredients to seafood products.

Research Lines:

- Rheological, structural, sensorial, enzymatic and thermal aspects related with food texture.
- Rheological characterization and texture optimization of frozen vegetables creams and purees.
- High-pressure effects on rheothermal properties and texture of vegetables creams and purees.
- Development, valorization and innovation of seafood products.

Skills:

- Rheological characterization and texture optimization of fresh and frozen vegetables. Processing and preservation effects; Product, process and preservation specifications.
- Objective measurements of the texture by fundamental methods based on mechanical properties of solids (compression, shear, relaxation stress, bend, creep-compliance, etc.) and rheology of semi-solids (oscillatory dynamic rheometry and static assays).
- Gelation of seafood muscle. Seafood muscle restructuration. Development of new products based in seafood muscle. High pressure.

Keywords: rheology, texture, structure, processing, foods, seafood, processing, restructured seafood products, high pressure

RESEARCH EXPERIENCE

Beatriz Herranz Hernández holds a PhD in Food Science and Technology from the Complutense University of Madrid (UCM) (2003), after developing

her PhD studies at the Department of "Nutrition, Bromatology and Food Technology" of Veterinary Faculty, in Madrid. She developed her research activities in this Department ("Nutrition, Bromatology and Food Technology") as assistant lecturer (2003-2006; 2008-2009), and later she continued her postdoctoral studies during one year (2006-2007) at Department of "Structuring Food for Health" of Institute of Food Research (IFR), United Kingdom. Finally, she continued her research career in her home institution "the Spanish National Research Council" (CSIC) at the Department of "Seafood valorization and health" (2009-2014) and "Characterization, Quality and Safety (DCCS)" (2014-2016) of Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), where she has worked since then. Her research activity has focused on the study of the addition of food ingredients and additives, such as hydrocolloids and amino acids, and/or using high hydrostatic pressure (HHP) in undervalued fish species and fishing industry byproducts, which presents lack of functionality due to previous processing or it has too much fat, through a rheological characterization in order to make restructured seafood products with an appropriate textural and sensorial characteristics. In particular, it was thoroughly investigated the influence of ingredient concentration and thermal conditions on the stability of the networks through a wide range of viscoelastic techniques relating to the physicochemical, mechanical and structural properties. These studies were carried out to determine the food ingredients gelation conditions most suited to making gels in combination with minced fish, with the appropriate texture for making restructured seafood products "on demand" according to consumer preferences. Moreover, it was studied the effect of high hydrostatic pressure (HHP) processing together with different ingredients and additives to enhance the gelation of low-quality surimi.

Overall, this has given place to 32 papers published in international scientific journals (included in the Journal Citation Reports® with high JCR impact factors) and 18 papers in divulging scientific journals, 1 patent, 6 book chapters, 39 works presented in scientific national and international meetings, and a PhD work in progress in her home institution. Her current project involves the rheological study through dynamic viscoelastic and steady-shear rheological techniques on the development of gluten-free products in the form of soups, purées and baked products made with different legume flours in order to try to mimic wheat-flour based products establishing optimum formulations and technological conditions that provide the desired functionality, nutritional value and sensory quality of these products.

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Chapter 2

BUCKWHEAT-CONTAINING GLUTEN-FREE BAKERY AND CONFECTIONERY PRODUCTS

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ABSTRACT

Coeliac disease is a permanent intolerance to gluten proteins of many common cereals such as wheat, rye, barley and oat. Therefore, coeliac patients must be on a strict long-life gluten-free diet, which is usually poor in some essential nutrients. Due to the limitation of some nutrients, the fortification of gluten-free products is required to obtain a balanced diet for coeliac patients. A growing number of studies have investigated the application of pseudocereals in the production of nutrient-rich gluten-free products such as bread, pasta and confectionery products.

This paper offers the overview of application of one of the most used pseudocereals in gluten-free bakery and confectionery formulations – buckwheat grain. It refers to the application of light and wholegrain buckwheat flour in different formulations to obtain added value products with described reological characteristics of dough and nutritional and functional benefits and sensory parameters of the final products. A number of different types of buckwheat-containing gluten-free bread was

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chosen as the most frequently used bakery products to be descriebed, as well as buckwheat-containing gluten-free cookies and creckers as the representatives of confectionery products.

Keywords: gluten-free products, buckwheat, bakery products, confectionery products

1. INTRODUCTION

Coeliac disease is a permanent intolerance to gluten proteins of many common cereals such as wheat, rye, barley and oat. Coeliac desease patients suffer from symptoms such as diarrhoea, weight loss, and iron, folate or vitamins B12 and D deficiencies (Woodward, 2007). The prevalence of coeliac disease is estimated at 1 for 100–200 inhabitants (Cook, Burt, Collett, Whitehead, Frampton, Chapman, 2000).

Coeliac disease patients are recommended to be on a strict long-life gluten-free diet, which implies using gluten-free ingredients such as gluten-free flours, starches, hydrocolloids, proteins and other gluten-free materials. Gluten-free foods frequently contain a greater density of fat and sugar than their gluten-containing counterparts (Kulai, Rashid, 2014), and, therefore, increased fat and calorie intake have been identified in individuals on a gluten-free diet (Zuccotti, Fabiano, Dilillo, Picca, Cravidi, Brambilla, 2013). Obesity, overweight, new-onset insulin resistance and metabolic syndrome have been identified in cealiac disease patients after initiation of a gluten-free diet (Kabbani, Goldberg, Kelly, Pallav, Tariq, Peer, Hansen, Dennis, 2012; Reilly, Aguilar, Hassid, Cheng, Defelice, Kazlow, Bhagat, Green, 2011; Tortora, Capone, De Stefano, Imperatore, Gerbino, Donetto, Monaco, Caporaso, Rispo, 2015). Gluten-free diet also lacks in certain essential nutrients (Thompson, Dennis, Higgins, Lee, Sharrett, 2005) and leads to some nutritional deficiencies among coeliac patients (proteins, dietary fibres, minerals, and vitamines) (Saturni, Ferretti, Bacchetti, 2010; Shepherd, Gibson, 2013; Wild, Robins, Burley, Howdle, 2010). Therefore, the fortification of gluten-free products is required to obtain a balanced diet for coeliac patients. There are many papers about gluten-free added value products, especially in the category of bakery and confectionery products (Alvarez-Jubete, Arendt, Gallagher, 2009; 2010; Alvarez-Jubete, Auty, Arendt, Gallagher, 2010; Matos, Sanz, Rosell, 2014; Sakač et al., 2015; Schoenlechner, Drausinger, Ottenschlaeger, Jurackova, Berghofer, 2010; Schoenlechner, Linsberger, Kaczyc, Berghofer,

2006; Torbica, Hadnađev, Dapčević-Hadnađev, 2012). These products are atractive not only for coeliac disease patients, but also for people who suffer from non-coeliac gluten sensitivity and those who decided to avoid gluten in their diet for lifestyle reasons (Brouns, van Buul, Shewry, 2013). The number of consumers who do not consume gluten because they believe that this type of diet represents a precondition for healthier life constantly increases.

Demand for gluten-free products grows and be followed by impressive producer efforts to enlarge the range of gluten-free products worldwide. Referring to reports of leading market research companies, the gluten-free industry enjoyed a growth of 136% from 2013 to 2015, reaching estimated sales of $11.6 billion in 2015 and is projected to grow at a compound annual growth rate of approximately 6% between 2015 and 2019.

This trend is supported by the scientists who have been doing their best to upgrade the knowledge about gluten-free products as the basis for overcoming problems which are known to arise from gluten-free production (lack of cohesiveness and elasticity of gluten-free batters/doughs, low product volume, poorness in sensory profile of gluten-free products – bad texture, poor flavour, colourless and pronounced firmness). The problems related to the elimantion of gluten from food formulations exist, because it is responsible for flour processing characteristics, which reflect on final product structure, mouthfeel, sensory acceptibility and shelf life (Gallagher, Gormley, Arendt, 2004; Giménez-Bastida, Piskuła, Zieliński, 2015). Therefore, the main goal is to find ingredients good enough to be a gluten substitute (to mimic its functional properties), and, if possible, to provide nutritional and/or functional benefit of gluten-free products. It is recommended to use a mixage of gluten-free ingredients (flours) rather than just a single component to be able to produce products with better sensory and textural properties (Arendt, Morrissey, Moore, Dal Bello, 2008; Capriles, Santos, Arêas, 2016).

2. BUCKWHEAT FLOUR

Pseudocereals are important representatives in the category of ingredients for gluten-free flour production, among which buckwheat is one of the most frequently used one. It belongs to the Polygonaceae family. The most widely grown buckwheat species are common buckwheat (*Fagopyrum esculen*tum) and tartary buckwheat (*Fagopyrum tataricum*). Buckwheat seed is used for production of two types of buckwheat flour – light (without hull) and dark (with hull) flour, both of which are used as the main ingredients in gluten-free

formulations, as well as the sources for their enrichment from the nutritional and functional point of view (Sakač, Sedej, Mandić, Mišan, 2012; 2015; Sedej, Mandić, Sakač, Mišan, Tumbas, 2010; Sedej, Sakač, Mandić, Mišan, Tumbas, Hadnađev, 2011; Torbica, Hadnađev, Dapčević-Hadnađev, 2012). The abundance of nutritional and functional ingredients originates from dehulled buckwheat groat, which containes 55% starch, 12% proteins, 4% lipids, 2% soluble carbohydrates, 7% total dietary fibres, 2% mineral matters, and 15% other compounds including organic acids, polyphenols, tannins, nucleotides and nucleic acids (Mandić, Sedej, Sakač, Mišan, 2013; Sakač, Sedej, Mandić, Mišan, 2015; Sedej, Sakač, Mandić, Mišan, Tumbas, Čanadanović-Brunet, 2012; Zhu, 2016).

Nutritional profile of buckwheat is high due to the well-balanced amino acid composition and high lysine content compared to cereals (Bonafaccia, Marocchini, Kreft, 2003; Kato, Kayashita, Tomotake, 2001). It containes high amount of essencial polyunsaturated fatty acids (PUFA), especially linoleic acid (18:2), while starch and dietary fibres are approximately at the same levels as in cereals (Brennan, 2005; Steadman, Burgoon, Lewis, Edwardson, Obendorf, 2001a). Buckwheat is characterized by the presence of some vitamines (B, C, and E), a wide range of minerals (Mg, Zn, K, P, Cu, and Mn are higher in buckwheat compared to cereals) (Steadman, Burgoon, Lewis, Edwardson, Obendorf, 2001b) and antioxidants (Zielińska, Zieliński, 2009). Its amino acid profile, abundance of minerals, dietary fibres, as well as a high amount of rutin, a potent antioxidant (Boots, Drent, De Boer, Bast, Haenen, 2011; Jiang, Burczynski, Campbell, Pierce, Austria, Briggs, 2007), quercetin and other phenolic compounds (Oomah, Mazza, 1996) guarantee that the enrichment of gluten-free products using buckwheat flour(s) can be achieved (Alvarez-Jubete, Wijngaard, Arendt, Gallagher, 2010; Sedej, Sakač, Mandić, Mišan, Tumbas, Hadnađev, 2011; Zhang et al., 2012; Zieliński, Michalska, Amigo-Benavent, del Castillo, Piskuła, 2009).

3. BUCKWHEAT-CONTAINING GLUTEN-FREE BREAD

Searching the literature on gluten-free bread production including the investigation of batter/dough and characterization of the final products results in a range of papers offering many aspects concerning recipes, analysed parameters and interpretation (and correlation) of the obtained results (Masure, Fierens, Delcour, 2016).

One of the possibilities to produce gluten-free bread is to use a single gluten-free flour in a bread formulation (Hager, Wolter, Czerny, Bez, Zannini, Arendt, Czerny, 2012). The mentioned authors investigated a range of gluten-free ingredients for breadmaking process including buckwheat flour. They revealed that 100% buckwheat-containing gluten-free bread had lower dough rise compared to wheat bread, but higher gas production, which indicated a more favourable sugar composition for yeast fermentation. Apart from higher gas production, buckwheat bread lacked in cohesive protein matrix and consequently its loaf volume was lower than for wheat bread. Concerning the texture, 100% buckwheat bread had a high value of crumb hardness in comparison to wheat bread, which can be related to an old product by the consumers. Its springiness was similar to wheat bread, but chewiness was worst among different types of gluten-free bread. As visual texture of the crumb represents an important attribute of bread quality, the above mentioned authors used digital image analysis to describe the crumb grain. The buckwheat bread possessed lower number of cells as a result of different dough consistency compared to wheat dough, with lower area of cells, which indicated a denser structure of buckwheat bread and resulted in poor specific volume of the buckwheat loaf. Its wall thickness, as a measure of mechanical strength of the bakery product, was slightly increased in relation to wheat bread. This reflects on mouthfeel of buckwheat bread, resulting in a harder and less elastic texture (Scalon, Zghal, 2001). In addition, buckwheat bread was assessed as less pronounced to staling (Hager, Wolter, Czerny, Bez, Zannini, Arendt, Czerny, 2012), but is belongs to a group of breads characterized by a higher water activity than wheat bread. It makes buckwheat bread prone to microbial spoilage and, therefore, its shelf life is predicted to be less than 4 days. Aroma profile of the buckwheat gluten-free bread was defined as pea-like, mouldy and viniger-like and positioned this type of bread much lower to that of wheat bread. In conclusion, although buckwheat flour is considered better than wheat flour in terms of nutritional quality, its breadmaking properties and resulting sensory characteristics of the obtained bread compromise its suitability for gluten-free bread production.

Therefore, its utilization requires mixing with two or more ingredients, which can lead to an improvement of the product (Alvarez-Jubete, Auty, Arendt, Gallagher, 2010; Hager, Arendt, 2013; Mezaize, Chevallier, Le-Bail, de Lamballerie, 2009; 2010; Miñarro, Normahomed, Guamis, Capellas, 2010; Peressini, Pin, Sensidoni, 2011; Sakač, Torbica, Sedej, Hadnađev, 2011; Torbica, Hadnađev, Dapčević, 2010; Wronkowska, Haros, Soral-Śmietana, 2013; Wronkowska, Zielińska, Szawara-Nowak, Troszyńska, Soral-Śmietana,

2010). Both types of buckwheat flour are used in creation of gluten-free breads with the aim to enrich the final product in proteins, minerals, dietary fibres and antioxidants and thereby to achieve minimal influence on its technological and sensory quality.

One of the possibilities in gluten-free breadmaking is using the mixture of rice and buckwheat flour. Rice flour is a frequently used component in gluten-free diet due to the absence of gluten, and it is favoured because of its bland taste, colourlessness, digestibility and hypoallergenic properties (Alvarez-Jubete, Auty, Arendt, Gallagher, 2010; Rossel, Marco, 2008), but its usage in breadmaking be followed by technological difficulties due to the absence of gluten (Sivaramakrishnan, Senge, Chattopadhyay, 2004). Most of the gluten-free products on rice flour basis contain starch and different types of hydrocolloids, but they are poor in nutrients and fibres (Torbica, Hadnađev, Dapčević, 2010), and, consequently, these products need to be upgraded with some functional ingredient(s), e.g., buckwheat flour (Prestamo, Pedrazuela, Penas, Lasuncion, Arroyo, 2003). The creation of optimal rice and buckwheat mixtures for gluten-free bread production (with good physicochemical behavior of dough) can be based on utilization of a rheological tool such is Mixolab, which measures dough behaviour during mixing and heating in a single test simulating kneading and baking processes. Torbica, Hadnađev, and Dapčević (2010) investigated Mixolab profiles of different rice-buckwheat mixtures comparing them with the Mixolab profile of the wheat dough. The idea was to create mixtures which would have as much as possible similar Mixolab profiles to the standard (wheat).

Mixolab parameter (C2 value), which represents the resistance of the dough to kneading at increasing temperature, was much higher in the case of rice flour compared to wheat flour and it became lower when buckwheat flour was incoroprated into the system. It is assumed that buckwheat flour has protein with lower quality characteristics from the technological point of view than rice flour and its presence in the dough reflects on protein-dependent baking characteristics leading to the lower score of the final product (Mariotti, Lucisano, Pagani, Iameti, 2008). The addition of buckwheat flour into the system also contributed to decreasing of the value of torque at C3 point and C5-C4 point, which means that gelling ability decreased with increasing amount of buckwheat flour in the system, because rice flour is superior concerning starch gelatinization compared to buckwheat flour. Also, C5-C4 value, as the measure of starch retrogradation degree, declined when higher amounts of buckwheat flour, especially unhusked flour, were incorporated in the investigated dough. This fact seems to be promising in terms of bread

staling, e.g., using of buckwheat flour in bread formulation can improve bread shelf life. This improvement is of special interest since gluten-free breads exhibit faster rate of staling in comparison to wheat breads (Kadan, Robinson, Thibodeaux, Pepperman, 2001).

Based on the Mixolab profiles it was concluded that utilization of husked buckwheat flour in the range of 10-30% in the rice flour dough system guaranteed its higher stability and stronger protein network structure, as well as higher peak viscosity compared to the system with unhusked buckwheat flour. Higher peak viscosity (C3 value), as a result of higher starch swelling (Debet, Gidley, 2006), corresponded with lower protein and lipid content in husked buckwheat flour. However, both husked and unhusked buckwheat flour can be successfully incorporated into the rice-containing gluten-free bread formulation up to 30%.

Marco and Rosell (2008) cited that mixtures of rice and buckwheat flour with hydrogenated vegetable fat possess the potential to give gluten-free breads with good sensory attributes. Therefore, Torbica, Hadnađev, and Dapčević (2010) prepared series of rice-buckwheat gluten-free breads containing both types of buckwheat flour in the range of 10-30% and evaluated their sensory scores.

Although the increase in the amount of unhusked buckwheat flour led to lower scores for the crust appearance and breadcrumb softness compared to breads with husked buckwheat flour, all evaluated breads were sensory acceptable following the mentioned parameters. Breads with husked buckwheat flour were characterized by more pleasant taste, because of the presence of aromatic compounds, which dominates in white buckwheat flour (Prosen, Kokalj, Janeš, Kreft, 2010). Breads containing unhusked buckwheat flour had bitter taste, which could be addressed to the compounds from husk (Luthar, 1992). All investigated breads were tested to hardness, which was not significantly different in all samples regardless the added amount of both types of buckwheat flour.

As buckwheat flour possesses a range of functional compounds, first of all antioxidants, Sakač, Torbica, Sedej, and Hadnađev (2011) decided to provide an insight into antixidant capacity of rice-buckwheat gluten-free breads. In addition, the influence of baking process on antioxidative components was also investigated, because processing conditions affect chemical composition of foods, e.g., baking negatively influences their antioxidative properties and polyphenol composition (Zieliński, Kozłowska, Lewczuk, 2001; Zieliński, Michalska, Amigo-Benavent, del Castillo, Piskuła, 2009).

During the breadmaking process rice-buckwheat gluten-free breads expressed different changes of antioxidative parameters (total phenolic content (TPC), antioxidant activity (AOA), reducing power, DPPH scavenging activity, and chelating activity on Fe^{2+}). Samples containing light buckwheat flour (LBF) exhibited decrease in TPC in comparison to calculated values, while determined values of TPC for wholegrain buckwheat flour (WBF) containing samples remained unchanged for the breads with 10% and 20% of WBF. However, the sample prepared with 30% of WBF expressed loss of about 17% of TPC. In comparison to calculated values, antioxidant and reducing activities showed decreasing trend by the addition of both buckwheat flours at 10% and 20%. These activities were slightly lower for the sample with 30% of LBF and practically unchanged for the sample with 30% of WBF. In comparison to calculated values, an increase in chelating and DPPH scavenging activity for all investigated samples was recorded. It was estimated that breads containing WBF showed two times higher activity than LBF-containing samples, because wholegrain buckwheat flour is superior in polyphenols compared to light buckwheat flour (Sedej, Sakač, Mandić, Mišan, Tumbas, Čanadanović-Brunet, 2012; Sedej, Sakač, Mandić, Mišan, Tumbas, Hadnađev, 2011; Şensoy, Rosen, Ho, Karwe, 2006). The registered increase in DPPH activity of gluten-free breads can be explaind by occuring the reactions of synthesis during heat tretament, i.e., formation of Maillard reaction products (Gawlik-Dziki, Dziki, Baraniak, Lin, 2009; Manzocco, Calligaris, Mastrocola, Nicoli, Lerici, 2001).

Table 1. Contents of rutin and quercetin in rice flour (RF), light (LBF) and wholegrain buckwheat flour (WBF) (*Sakač, Torbica, Sedej, Hadnađev, 2011*)

Material	Rutin (mg/100 g d.m.)	Quercetin (mg/100 g d.m.)
RF	n.d.[a]	n.d.[a]
LBF	8.71 ± 0.12[b]	0.54 ± 0.05[b]
WBF	21.34 ± 0.05[c]	0.58 ± 0.075[b]

Values are means of three determinations ± standard deviation.

Values of the same column with the same superscript are not statistically different ($P < 0.05$).

n.d. – not detected; d.m. – dry matter

The determined antioxidant capacity of the investigated types of bread is the consequence of the antioxidant activities of phenolic compounds identified in both types of buckwheat flour – rutin, quercetin and other polyphenolics. Contents of rutin and quercetin in RF, LBF and WBF are presented in Table 1.

Due to a higher amount of rutin in WBF than in LBF (Dietrych-Szostak, Oleszek, 1999; Sedej, Sakač, Mandić, Mišan, Tumbas, Hadnađev, 2011; Sedej, Sakač, Mandić, Mišan, Tumbas, Čanadanović-Brunet, 2012) its content in rice-buckwheat gluten-free breads with WBF was higher than in LBF-containing breads. In addition, increasing amount of both types of buckwheat flour in the mixture for gluten-free bread resulted in increase in rutin and quercetin in the produced breads. However, the polyphenol pattern of gluten-free breads is changed in comparison to flours during breadmaking process (Figure 1). Despite the fact that loss of some functional components in buckwheat is evidenced due to the thermal treatment (Şensoy, Rosen, Ho, Karwe, 2006), Figure 1 ilustrates the loss of rutin during baking, but the tremendous increase in quercetin. The loss of rutin content in breads with 20% and 30% of LBF and WBF was in the range from 26% to 40%, while quercetin content increased 1.5 to 7 times, probably due to hidrolysis of rutin to quercetin.

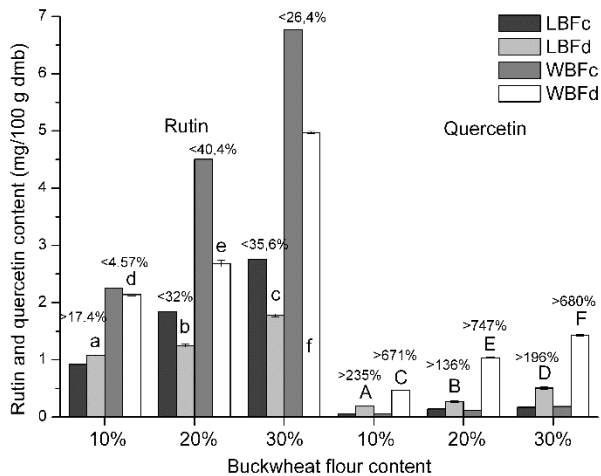

Figure 1. Changes in rutin and quercetin contents in gluten-free breads; LBFc — calculated content in light buckwheat bread, LBFd — determined content in light buckwheat bread, WBFc — calculated content in wholegrain buckwheat bread, WBFd — determined content in wholegrain buckwheat bread; and </>% — mean loss/yield% (*Sakač, Torbica, Sedej, Hadnađev, 2011*).

In addition to the above mentioned example of the rice-buckwheat-containing gluten-free bread formulation and its antioxidant capacity, the paper published by Alvarez-Jubete, Arendt and Gallagher (2009) presents the incorporation of psedocereal flours (including buckwheat flour) into a rice flour-potato starch based gluten-free bread formulation, which was followed by the improvement of its nutritional profile. The 50% replacement of potato starch by buckwheat flour resulted in bread with higher nutrient content compared to the gluten-free control (based on refined flours and starches) in terms of protein, fat, ash and dietary fibres, while starch content became lower. Not only the higher protein content of buckwheat-containing gluten-free bread represents a special nutritive feature but also its amino acid profile, which classifies this type of bread in a superior category (high in lysine and low in glutamic acid and proline). Digestibility of buckwheat proteins is relatively low and this fact is considered to be partly responsible for cholesterol-lowering effect of buckwheat-containing food (Li, Zhang, 2001). Another important feature of buckwheat nutrient composition is related to lipids, which are characterized by a high degree of unsaturation, resulting in a desirable fatty acid profile (with linoleic, oleic and palmitic acid as the main fatty acids) of the produced buckwheat-containing gluten-free bread (Alvarez-Jubete, Arendt, Gallagher, 2009). Being a rich source of minerals, especially magnesium, zinc, copper, manganese and iron (Steadman, Burgoon, Lewis, Edwardson, Obendorf, 2001b), buckwheat flour ensured significantly higher mineral content in the investigated bread formulation compared to the gluten-free control (Alvarez-Jubete, Arendt, Gallagher, 2009). Therefore, the mineral deficiency in a gluten-free diet can be overcome by using buckwheat flour for achieving gluten-free bread improvement. Besides being important from the mentioned point of view, buckwheat flour offers other bioactive compounds such as fagopyritols and polyphenols (Giménez-Bastida, Zieliński, 2015; Holasova, Fiedlerova, Smrcinova, Orsak, Lachman, Vavreinova, 2002; Sakač, Sedej, Mandić, Mišan, 2012; 2015; Sedej, Sakač, Mandić, Mišan, Tumbas, Čanadanović-Brunet, 2012; Sedej, Sakač, Mandić, Mišan, Tumbas, Hadnađev, 2011), phytosterols (Cai, Corke, Li, Colin, 2004), as well as vitamin E (Alvarez-Jubete, Holse, Hansen, Arendt, Gallagher, 2009).

The buckwheat-containing gluten-free bread was also investigated regarding its baking properties and texture profile and compared with the gluten-free control (Alvarez-Jubete, Auty, Arendt, Gallagher, 2009). Loaf volume was increased when buckwheat flour was used to replace potato starch in the gluten-free bread formulation. This bread possessed darker crust colour (lower L* value), which is desirable, because gluten-free breads are usually

considered too light (Gallagher, Gormley, Arendt, 2003). The buckwheat-containing gluten-free bread had a softer and more cohesive crumb, as well as higher crumb springiness in comparison with the control. The softer crumb was addressed to the presence of the emulsifiers naturally present in the buckwheat flour (Alvarez-Jubete, Auty, Arendt, Gallagher, 2009). The fact that buckwheat flour gave a softer texture to the produced bread represents a desirable characteristic as gluten-free breads often have hard texture. The increased cohesiveness and springiness found in bucwheat-containing bread can be considered beneficial, because the texture of many gluten-free breads is known to be crumbly and brittle. Incorporation of the buckwheat flour in the gluten-free bread formulation did not affect sensory properties of the loaves compared to the control. This finding was in line with slightly reduced overall sensory score detected for the gluten-free bread containing wholegrain buckwheat flour and potato starch (64%:36%) in comparison to bread containing rice flour and potato starch (50%:50%) or 100% rice bread (Capriles, Santos, Reis, Pereira, 2015). The decreased score for bread prepared with 64% buckwheat flour resuled from its lower acceptibility score for colour. The storage of the buckwheat-containing gluten-free bread resulted in an increase in crumb hardness and a decrease in the moisture content, especially after 120 h (Alvarez-Jubete, Auty, Arendt, Gallagher, 2009).

The paper published by Alvarez-Jubete, Arendt and Gallagher (2009) also discovers the possibility to use sprouted buckwheat flour as an ingredient in gluten-free bread formulation. Sprouting is connducted to improve protein and starch digestion, increase sugars, vitamins, and antioxidant content and decrease phytates (Sakač, Sedej, Mandić, Mišan, 2012), i.e., to improve nutritional quality of the seeds. The authors also underlined that buckwheat flour is a potent component for designing products with low glycaemic index, which is desirable in coeliac patient diets (Wolter, Hager, Zannini, Arendt, 2013). Low glycemic index of buckwheat flour is a consequence of its starch content (Skrabanja, Elmstahl, Kreft, Bjorck, 2001), which is represented by resistant starch in amount of 33.5-37.8% (Skrabanja, Laerke, Kreft, 1998).

Besides presented results obtained in gluten-free breadmaking by mixing buckwheat flour with rice-based gluten-free mixture, there was an attempt to produce gluten-free bread by mixing buckwheat flour (up to 40%) with corn starch-based gluten-free mixture (Wronkowska, Zielińska, Szawara-Nowak, Troszyńska, Soral-Śmietana, 2010). The authors aimed to develop a new formula for buckwheat-enriched gluten-free bread with increased antioxidant capacity, rich in minerals and with high sensory score. The antioxidant capacity of buckwheat-enriched gluten-free breads showed dose-dependent-

buckwheat flour substitute effect, e.g., the bread containing 40% of common buckwheat flour had the highest TPC content, as well as the most potent scavenging activity on DPPH· and $ABTS^{+}$·. Also, increasing amounts of macroelements (calcium, magnesium, phosphorus, and potassium) were detected in gluten-free breads. In comparison with the control bread, volume of all produced buckwheat-containing breads was considerably higher as it was previously noticed by Alvarez-Jubete, Auty, Arendt, and Gallagher (2009). The positive effect of buckwheat flour on the loaf volume of gluten-free bread was also confirmed by Wronkowska, Haros, and Soral-Śmietana (2013). The differences in loaf specific volume between buckwheat-containing gluten-free bread and those containing potato or corn starch can be partly explained by the differences in water-binding capacity of the ingredients.

The average overall scores for buckwheat-containing breads pointed to the best sensory profile of the bread with 40% of buckwheat flour, whereas the control bread obtained the lowest score. The authors underlined that in the buckwheat-containing breads "buckwheat" attribute dominated, being accompanied by bitterness. In addition, the obtained results pointed to the colour, springiness, elasticity and moistness of the breads with buckwheat flour. The breads containing higher amounts of buckwheat flour had a higher moistness, and, consequently, kept softness during storage.

The importance of above mentioned sensory parameters was confirmed when the principal component analysis (PCA) was performed. Considering the map of the PCA performed on the sensory data, colour, buckwheat odour, bitter taste, buckwheat taste, springiness, elasticity, and moistness exhibited positive scores according to the first principal component (PC1), which contains the most important information and includes more important characteristics. Rancid odour, rancid taste and gumminess showed negative score values according to the PC1. The negative contribution to the second principal component (PC2) was observed for yeast odour and taste. Additionally, PCA technique differentiated the samples by kind of breads, locating them in separate clusters along the first and second principal component. As the result of PCA, the control and 10% buckwheat-containing gluten-free bread were located oppositely to 20%, 30% and 40% buckwheat-containing breads.

The results published by Wronkowska, Zielińska, Szawara-Nowak, Troszyńska, and Soral-Śmietana (2010) were in agreement with those determined by Torbica, Hadnađev, and Dapčević (2010), who descovered that increasing amounts of husked buckwheat flour (10-30%) in the bread formulation be followed by increased scores for taste of the rice-buckwheat-

containing gluten-free breads, whereas the scores for appieriance, softness, and flavour did not differ significantly.

Wronkowska, Haros, and Soral-Śmietana (2013) investigated the same gluten-free breads (obtained by the substitution of corn starch in the gluten-free bread formulation using buckwheat flour in the range of 10-40%) using image analysis and found better crumb structure in breads with increasing amount of buckwheat flour in comparison to the control bread. Contrary, their crumb hardness decreased with increasing amount of buckwheat flour, but became higher during a 72-h storage. In the case of gluten-free bread with the highest amount of buckwheat flour (40%) the significant decrease in hardness was noticed at the end of the 72-h storage. This finding was in agreement with the decrease in enthalpy of starch gelatinization associated to amylopectin recrystallization responsible for braed staling.

The addition of buckwheat flour in the gluten-free bread formulation influenced the crumb and crust colour (Wronkowska, Haros, Soral-Śmietana, 2013). The crust colour showed a decrease in whiteness and had significantly increased browing index with increasing amount of buckwheat flour in the bread formulation, which can be considered desirable from the consumer's point of view. Regarding the colour, the authors classified the investigated gluten-free breads in the category close to whole wheat bread.

Although buckwheat flour is well known as an ingredient for supplementation of gluten-free bread formulations, a trend for fortification of buckwheat-containing gluten-free bread formulation exists. Costantini, Lukšič, Molinari, Kreft, Bonafaccia, Manzi, and Merendino (2014) used chia seed flour to fortify tartary and common buckwheat flour in a gluten-free bread formulation. The reason for using chia seed flour is its abundance in omega-3 alpha-linolenic acid (up to 68%), as well as a good balance between omega-6 and omega-3 fatty acids (Ayerza, Coates, 2011). Choosing the tartary buckwheat flour as the main ingredient in gluten-free bread formulation is supported by the fact that tartary buckwheat possesses a better nutritional and functional profile than common buckwheat (Bonafaccia, Marocchini, Kreft, 2003), with the exception regarding its bitter taste related to high rutin content. The results obtained in the study of the mentioned authors indicated that the combination of chia flour with tartary buckwheat flour led to the creation of bread with enhanced functional properties without adversely affecting the investigated physical characteristics (loaf volume, specific volume and colour) in comparison to the control. The incorporation of chia flour slightly increased the specific volume of buckwheat-containing bread, but did not significantly affect colour parameters. Chia flour-supplemented breads (with 10% of chia

seed flour) contained significantly higher amounts of proteins, insoluble dietary fibres, ash and especially omega-3 fatty acids. Tartary buckwheat flour possessing a high amount of flavonoids (with remarkable antioxidant capacity) was recognized to be suitable for prevention of lipid oxidation in chia-containing gluten-free bread.

4. BUCKWHEAT-CONTAINING GLUTEN-FREE BREADS WITH HYDROCOLLOIDS

Gluten is a structure-building protein that provides dough viscoelasticity, gas-holding ability and good crumb structure of the baked product. Therefore, the main goal in gluten-free bread production is to mimic gluten properties using gums, hydrocolloids and protein-based ingredients (Arendt, Morrisey, Moore, Dal Bello, 2008). There is an increased number of studies on gluten-free breads with the addition of hydrocolloids, as well as other ingredients (different starches, non-gluten proteins, dairy products, etc.) for improving their structure, sensory properties and shelf life. Hydrocolloids are used in gluten-free formulations to modify functionality of proteins from gluten-free flours in order to improve baking characteristics by promoting protein networks.

The inclusion of gums, pectin, carboxymethylcellulose, agarose, xanthan or oat β-glucan into the gluten-free formulation was done to improve the specific volume of gluten-free bread made of rice flour (Lazaridou, Duta, Papageorgiou, Belc, Biliaderis, 2007).

Xanthan gum (XG) was used as a gluten replacement in the development of gluten-free bread improving dough elasticity (Demirkesen, Mert, Sumnu, Sahin, 2010). Several studies have been carried out showing the effects of XG on gluten-free breads (Lazaridou, Duta, Papageorgiou, Belc, Biliaderis, 2007; Moore, Schober, Dockery, Arendt, 2004; Schober, Messerschmidt, Bean, Park, Arendt, 2005).

Moore, Schober, Dockery, and Arendt (2004) developed the gluten-free bread formulation based on corn starch, brown rice, soya, buckwheat flour and xanthan gum, but the loaf volume of the produced bread was lower compared to the control made of the commercial flour mixture and it was brittle after two days of storage.

Xanthan gum was also used in flour-based (buckwheat-containing) gluten-free recipe and the obtained bread had lower specific volume than other breads

based on starch or starch-vegetable ingredients (Miñarro, Normahomed, Guamis, Capellas, 2010). It is assumed that non-endosperm components (germ, bran, and epicarp hairs), which are present in buckwheat flour, are responsible for producing low volume by disrupting dough structure. Also, the addition of buckwheat flour into the mentioned gluten-free bread formulation be followed by lower bake loss due to high fibre content, which absorbed water and help in its retention during baking. However, buckwheat-containing bread was characterized by inferior texture profile, i.e., higher crumb firmness and lower cohesiveness. The colour of this type of gluten-free bread was darker, i.e., L* value was lower and a* value was higher in comparison with other investigated types of gluten-free bread.

The effects of hydrocolloid addition (xanthan gum and propylene glycol alginate) on rheological properties and breadmaking performance of rice-buckwheat batter (60:40) was tested at different water levels (Peressini, Pin, Sensidoni, 2011). Hydrocolloids were added at the levels of 0.5-1.5%. The incorporation of PGA gave the best effect in terms of rheological properties due to the combined effect of low batter viscosity and elasticity developed by the polymer and the ability to form elastic films at the gas-liquid interface, which protect gas cells from instability. Breadmaking performances of rice-buckwheat flour mixture can be successfully improved by the addition of both xanthan gum (XG) and propylene glycol alginate (PGA). PGA provided breads with higher quality regarding specific volume, crumb mechanical properties and crumb structure than XG.

Alvarez-Jubete, Auty, Arendt, and Gallagher (2010) recommended the incorporation of hydroxypropylmethylcellulose (HPMC) in buckwheat-containing gluten-free formulations to result in breads with improved crumb structure and volume. It provides gas retention of dough during the fermentation step (Ylimaki, Hawrysh, Hardin, Thomson, 1991) and dough characteristics similar to those of wheat dough (Sivaramakrishnan, Senge, Chattopadhyay, 2004).

The influence of HPMC and xanthan and their combination on gluten-free model systems (including buckwheat-containing system) was also investigated (Hager, Arendt, 2013), namely loaf specific volume, crumb hardness, area of cells and and wall thickness were determined for the mentioned purpose. The significant effect was found of HPMC and water level on specific volume of buckwheat gluten-free bread. Increase of water addition had a positive linear effect, while HPMC had no effect on the bread volume. Contrary, xanthan addition had a negative effect on the loaf volume. The combination of HPMC and xanthan decreased specific volume of the buckwheat-containing bread. In

conclusion, according to Hager and Arendt (2013) and other authors (Peressini, Sensidoni, 2009), xanthan increases volume of gluten-free breads only at low levels (up to 0.5%) and HPMC is more suitable for the loaf volume increase (Mezaize, Chevallier, Le-Bail, de Lamballerie, 2009). Hager and Arendt (2013) explaned the reduction of loaf volume of xanthan-containing bread by increased viscosity of the batter and higher temperatures required for the formation of a starch gel compared to wheat bread, resulting in a longer time period in which bubbles expand due to heat (Schober, 2009).

As the crumb hardness represents the important textural characteristic of bread, the influence of the addition of HPMC and xanthan on that parameter was tested. The increase of water levels showed a negative linear effect, decreasing crumb hardness, and HPMC addition decreased this parameter too. The improving properties of HPMC on loaf volume are reported by Crockett, Ie, and Vodovotz (2011). Contrary, the crumb hardness was increased by xanthan addition. This observation was also noted by Lazaridou, Duta, Papageorgiou, Belc, and Biliaderis (2007) and Peressini and Sensidoni (2009). At constant water level HPMC and xanthan addition increased hardness of buckwheat bread.

It is known that mouthfeel is strongly influenced by cell characteristics – finer, thin-walled uniform cells yeald a softer and more elastic texture (Scalon, Zghal, 2001). Therefore, area of cells and wall thickness were investigated and the results pointed to a negative effect of water on area of cells, while HPMC increased the ratio. Mezaize, Chavallier, La Bail, and de Lamballerie (2009) observed that HPMC addition (2.3%) increased the number of cells per cm^2 and reduced bread porosity. Addition of HPMC to the buckwheat bread formulation resulted in an initial decrease of wall thickness, which became thicker at higher levels of HPMC (> 1.5%) (Hager, Arendt, 2013). A negative effect of xanthan and water level on area of cell was observed, but regarding the wall thickness, water addition showed a positive effect and xanthan addition had the opposite effect, decreasing wall thickness of buckwheat bread. When both hydrocolloids were used for buckwheat formulation, xanthan addition initially decreased the area of cells, which was increased at xanthan levels of around 2%. HPMC had no effect on the area of cells of buckwheat bread. Wall thickness was not influenced by using the combination of HPMC and xanthan.

In their experiment, Hager and Arendt (2013) optimized the formulation of buckwheat bread with HPMC, xanthan and both hydrocolloids, obtaining formulations: 1.50% for HPMC, 0.52% for xanthan, and 0.14% of xanthan without HPMC addition in the case of using both hydrocolloids in the braed

formulation. Results obtained by Hager and Arendt (2013) showed that both HPMC and xanthan are powerful ingredients that influence buckwheat-containing bread properties.

Mezaize, Chavallier, La Bail, and de Lamballerie (2009) developed and optimized gluten-free bread formulation of bread similar to French-style breads using different hydrocolloids (carboxymethylcellulose (CMC), guar gum, HPMC, and xanthan gum) and protein sources. Hydrocolloid addition had a significant impact on bread specific volume, crumb hardness, crust colour, crumb hardness, and gas cell size distribution when compared to the control and French breads. Specific volume was increased by guar gum and HPMC. Breads with guar gum had colour characteristics similar to French bread. Hardness decreased with the addition of hydrocolloids, especially HPMC and guar. The most heterogeneous cell size distribution was achieved using guar gum. Therefore, the gluten-free bread formulation including guar gum and buckwheat flour was investigated to conclude that 1.9% of guar gum (w/w, total flour basis) and 5% of buckwheat flour could be considered to mimic French bread quality attributes. Similar gluten-free bread formulation (based on rice and corn flour, corn and potato starch, as well as buckwheat flour with the addition of guar gum) was tested to get an insight into the influence of freezing on dough rheological properties and bread quality (Mezaize, Chavallier, La Bail, de Lamballerie, 2009). The introduction of freezing step in the breadmaking process had a negative impact on the gluten-free bread characteristics leading to denser breads, harder crumb with homogenous gas cells distribution, and modified crust colour.

Two commercial gluten-free bread mixtures were supplemented by a dehulled buckwheat flour (up to 40%), a puffed buckwheat flour and HPMC to evaluate the effects of their addition on the breadmaking performances (Mariotti, Pagani, Lucisano, 2013). The addition of the dehulled buckwheat flour improved the baking properties of the commercial gluten-free mixtures. The increase in loaf specific volume was detected using HPMC in a gluten-free mixture with corn starch, skimmed milk powder, sugar, Psyllium fibre, guar gum, and maltodextrines, but the addition of buckwheat flour in the same mixture did not affect loaf volume. The combination of 0.5% HMPC and 40% buckwheat flour resulted in the significant increase in specific volume and great decrease in crumb hardness. When puffed buckwheat flour was also included in the mixture, further improvements were obtained. Generally, the presence of high levels of buckwheat flour did not decrease the baking performances of the commercial gluten-free mixture, but upgraded its nutritional value. The colour of the final products was changed in terms of L*

and b*, too. In the case of gluten-free mixture contained corn starch, tapioca starch, potato starch, rice flour, sugar and salt, the incorporation of 0.5% HPMC and 40% buckwheat flour also resulted in the increased volume, while crumb moisture and softness were at the same level as in the control mixture. The significant improvement regards the nutritional profile was achieved using buckwheat flour, because the mixture was mainly starch-based.

5. BUCKWHEAT-CONTAINING GLUTEN-FREE CRACKERS

Crackers are popular snack products in the diet because of the low level of moisture, which leaves no medium for mould growth. On the other hand, they belong to a group of bakery products which contains fat in considerable amount, up to 30% (w/w) (Sedej, Sakač, Mandić, Mišan, Pestorić, Šimurina, Čanadanović-Brunet, 2011). Because of that, manufacturers are moving to meet consumer demands for healthier foods by providing an increased variety of low-fat and convenient options. Consumer desire for healthy snacks is resulting in an increasing variety of products that are low-fat, low-salt, and low- or no-cholesterol. There is also increasing demand for gluten-free crackers.

One of the possible ways to increase the nutritive value of gluten-free crackers is by using buckwheat in their formulation. The absence of structure-forming gluten proteins in buckwheat may result in poor resistance of dough (Pruska-Kedzior, Kedzior, Goracy, Pietrowska, Przybylska, Spychalska, 2008) and low quality of the final product. Therefore, the production of high-quality gluten-free products represents a significant technological challenge (Gallagher, Gormley, Arendt, 2004).

Gluten is the main structure-forming protein in flour, and is responsible for the elastic characteristics of dough, and contributes to the appearance and crumb structure of many baked products. Gluten removal results in major problems for bakers, products of low quality exhibiting poor mouthfeel and flavour. For that reason additional ingredients must often be used to compensate for a lack of these properties in gluten-free cracker products (Han, Janz, Gerlat, 2010). The use of pre-gelatinized rice starch, modified starch, xanthan gum, carboxymethylcellulose, hydroxypropylmethylcellulose, hydrocolloids, surfactants, and maltodextrin were tested by Han, Janz, and Gerlat (2010) in the formulation of gluten-free pulse crackers. The authors

found that the effect of these ingredients was minimal. Gluten-free pulse crackers formulated without the baking additives were of reasonable quality (Han, Janz, Gerlat, 2010).

In contrast to bread, in crackers the gluten network needs to be only slightly developed for the dough to be cohesive without being too elastic. Sedej, Sakač, Mandić, Mišan, Pestorić, Šimurina, and Čanadanović-Brunet (2011) managed to formulate gluten-free crackers from buckwheat flours, without the use of baking additives and showed that crackers made are significantly higher in nutrients in comparison with wheat crackers made from refined and wholegrain wheat flours. First, buckwheat crackers were formulated without wheat flour, using refined and whole buckwheat flour. The same formulation was applied for wheat flour crackers for comparison, using wholegrain and refined wheat flour (Table 2).

Table 2. The formulations of crackers (*Sedej, Sakač, Mandić, Mišan, Pestorić, Šimurina, Čanadanović-Brunet, 2011*)

Ingredient (g)	Refined wheat cracker	Refined buckwheat cracker	Wholegrain wheat cracker	Wholegrain buckwheat cracker
Refined wheat flour	70	0	0	0
Refined buckwheat flour	0	70	0	0
Wholegrain wheat flour	0	0	70	0
Wholegrain buckwheat flour	0	0	0	70
Cornmeal	30	30	30	30
Soy lecithin	1	1	1	1
Baking powder	1	1	1	1
Powdered sugar	2.5	2.5	2.5	2.5
Salt	3.5	3.5	3.5	3.5
Vegetable fat	30	30	30	30
Soaked flax seed	10	10	10	10
Sesame	10	10	10	10
Water	50	50	50	50

Table 3. Proximate composition of crackers
(*Sedej, Sakač, Mandić, Mišan, Pestorić, Šimurina, Čanadanović-Brunet, 2011*)

*Component (g/100 g)	Refined wheat cracker	Refined buckwheat cracker	Wholegrain wheat cracker	Wholegrain buckwheat cracker
Ash	3.60 ± 0.05^{b}	3.99 ± 0.08^{a}	3.92 ± 0.01^{a}	4.49 ± 0.18^{c}
Protein	10.9 ± 0.05^{a}	10.2 ± 0.18^{b}	10.5 ± 0.19^{a}	11.4 ± 0.04^{c}
Fat	25.3 ± 0.14^{a}	25.2 ± 0.16^{a}	25.7 ± 0.13^{b}	27.2 ± 0.27^{c}
Starch	53.1 ± 0.21^{d}	52.3 ± 0.11^{c}	48.9 ± 0.13^{b}	46.7 ± 0.84^{a}
Reducing sugar	3.47 ± 0.04^{c}	2.29 ± 0.15^{a}	2.86 ± 0.18^{b}	2.30 ± 0.16^{a}
Total dietary fiber	8.09 ± 0.06^{a}	9.28 ± 0.02^{b}	10.8 ± 0.08^{c}	11.8 ± 0.08^{d}

* Results are presented on dry weight basis. Values are means of three determinations ± SD.
Values in each row with the same superscript are not significantly different ($P < 0.05$).

Buckwheat crackers (both refined and wholegrain) were shown to contain higher content of ash in comparison to wheat ones (Table 3). Also, the protein content of the wholegrain buckwheat crackers was reported to be significantly higher ($P < 0.05$) in comparison to the wholegrain wheat crackers. Those results were in line with previous findings related to higher protein and mineral content of buckwheat flour in comparison with wheat flour (Bonafaccia, Gambelli, Fabjan, Kreft, 2003; Skrabanja, Kreft, Golob, Modic, Ikeda, Ikeda, Kreft, Bonafaccia, Knapp, Kosmelj, 2004). The protein content of the refined buckwheat crackers was lower in comparison with the refined wheat crackers, which was explained by the lower protein content of refined buckwheat flour in comparison with refined wheat flour used in the formulation. The content of the reducing sugar and starch was significantly higher ($P < 0.05$) in the refined and wholegrain wheat crackers than in their buckwheat cracker counterparts. Furthermore, the fat content of the wholegrain buckwheat cracker was significantly higher ($P < 0.05$) in comparison to the wholegrain wheat cracker. Total dietary fibre contents were higher in buckwheat crackers than in their wheat counterparts.

Sedej, Sakač, Mandić, Mišan, Pestorić, Šimurina, and Čanadanović-Brunet (2011) also compared phenolic profile, total phenolic content and antioxidant activity of the crackers. Strong antioxidant activity of the wholegrain crackers, measured by DPPH radical scavenging activity, was reported to be related to their higher phenolic content compared to the crackers made with refined flour (Table 4). Referring to the authors, the crackers formulated with buckwheat flour possessed around 2–3-fold higher total phenolic content when compared to the crackers made from wheat flour. Regarding phenolic profile, protocatechuic and ferulic acid were quantified in all tested crackers, whereas two flavonoids, rutin and quercetin only in buckwheat crackers. Higher rutin and quercetin content in wholegrain buckwheat crackers were connected with the higher ratio of hulls content in wholegrain than in refined buckwheat flour (Quettier-Deleu, Gressier, Vasseur, Dine, Brunet, Luyckx, Cazin, Cazin, Bailleul, Trotin, 2000).

Apart from phenolic compounds, the content of another group of strong natural antioxidants - tocopherols in the crackers was compared by Sedej, Sakač, Mandić, Mišan, Pestorić, Šimurina, and Čanadanović-Brunet (2011), because it is known that grains are relatively good sources of vitamin E, α-, β-, γ-, δ-tocopherols and tocotrienols. The content of total tocopherols in crackers ranged from 5.41 mg/100 g for refined wheat cracker to 6.99 mg/100 g for

wholegrain buckwheat cracker. α-, γ-, δ-Tocopherols in crackers were found in the following order: α- >> γ- > δ-tocopherol for all samples. The highest content of α-tocopherol was found in wholegrain buckwheat crackers. Content of γ-tocopherol was significantly higher in both buckwheat crackers in comparison to wheat ones. According to the authors, cornmeal, flax seed and sesame, as ingredients in the cracker formulation could have contributed to the total content of tocopherols in crackers. The authors also stated that the content of tocopherols strongly depended on the type of flour (refined or wholegrain) used in crackers production, namely refining process of flour, in which the outer layer of grain is removed. Within the grain, tocopherols are primarily located in the germ and aleurone tissue, the metabolically active parts of grain (Engelsten, Hansen, 2009). Therefore, higher content was found in wholegrain than in refined crackers.

The sensory quality of the crackers was defined based on evaluation of appearance, texture and aroma and the quality category was determined in dependence of scores: unacceptable (< 2.5), good (2.5–3.5), very good (3.5–4.5) and excellent (> 4.5) (Table 5).

According to the authors, sensory quality of the crackers indicated that buckwheat flours may be used in cracker formulations without producing a negative impact on the sensory properties. The authors noted that odour of crackers made of refined and wholegrain buckwheat flour was not significantly different from standard crackers' odour made of refined and wholegrain wheat flour.

The results of Sedej, Sakač, Mandić, Mišan, Pestorić, Šimurina, and Čanadanović-Brunet (2011) can be considered encouraging for the production of gluten-free crackers based on buckwheat flours. Such products would increase the diversity of functional bakery products and, even more importantly, of functional foods suitable for coeliac disease patients.

Table 4. Total phenolic content, phenolic compounds of crackers and scavenging activity on DPPH·
(*Sedej, Sakač, Mandić, Mišan, Pestorić, Šimurina, Čanadanović-Brunet, 2011*)

	Refined wheat cracker	**Refined buckwheat cracker**	**Wholegrain wheat cracker**	**Wholegrain buckwheat cracker**
Total phenolic content (mg GAE/100 g)	84 ± 1^{a}	231 ± 1^{c}	143 ± 3^{b}	292 ± 0^{d}
Phenolic compounds (mg/100 g)				
Protocatechuic acid	0.29 ± 0.01^{a}	1.59 ± 0.03^{c}	0.55 ± 0.01^{b}	2.42 ± 0.05^{d}
Ferulic acid	0.53 ± 0.00^{a}	0.69 ± 0.01^{b}	1.11 ± 0.01^{d}	0.75 ± 0.00^{c}
Rutin	n.d.	2.06 ± 0.05^{a}	n.d.	5.02 ± 0.04^{b}
Quercetin	n.d.	0.26 ± 0.01^{a}	n.d.	0.91 ± 0.06^{b}
Scavenging activity on DPPH· (IC_{50} mg/mL)	28.2 ± 1.17^{c}	1.63 ± 0.07^{a}	16.2 ± 1.85^{b}	0.95 ± 0.17^{a}

Values are means of three determinations ± SD.

Values in each row with the same superscript are not significantly different ($P < 0.05$).

Table 5. Sensory scores of crackers
(*Sedej, Sakač, Mandić, Mišan, Pestorić, Šimurina and Čanadanović-Brunet, 2011*)

Property	**Refined wheat cracker**	**Refined buckwheat cracker**	**Wholegrain wheat cracker**	**Wholegrain buckwheat cracker**
Appearance (shape, uniformity, surface)	3.97 ± 0.40^{b}	4.57 ± 0.40^{a}	4.66 ± 0.44^{a}	4.37 ± 0.39^{ab}
Texture				
Structure, break, firmness	4.49 ± 0.79^{a}	5.79 ± 0.28^{b}	5.25 ± 0.52^{ab}	5.14 ± 0.65^{ab}
Chewiness and other textural properties	3.57 ± 0.69^{a}	4.46 ± 0.51^{b}	3.97 ± 0.37^{ab}	3.83 ± 0.66^{ab}
Aroma				
Odour	3.25 ± 0.67^{a}	3.18 ± 0.67^{a}	3.18 ± 0.58^{a}	3.27 ± 0.68^{a}
Taste	4.42 ± 1.04^{a}	4.83 ± 1.41^{a}	4.70 ± 0.93^{a}	4.39 ± 1.47^{a}
Weighted mean value	3.94	4.57	4.35	4.2

Scores are means of seven evaluations by seven panelists ± SD.
Values in each row with the same superscript are not significantly different ($P < 0.05$).
Scores: 1 – unacceptable, 2 – acceptable, 3 – good, 4 – very good, 5 – excellent.

6. BUCKWHEAT-CONTAINING GLUTEN-FREE COOKIES

Cookies or biscuits present on the market could be found in various combinations of texture and taste, making them appealing to a widest range of consumers. Formulation of majority of commercially available gluten-free cookies is based on starches and starchy flours, which cause dry and sandy mouthfeel and crumbly texture. In order to achieve good taste and appearance of cookies similar to wheat flour cookies, the latest efforts are oriented towards formulation of gluten-free cookies based on gluten-free mixtures of corn and potato starches, rice and soy flour, millet flakes and pseudocereal flours including buckwheat flour.

Basic compounds in a cookie formulation are flour, water, sugar, fat and salt, and variations in ingredients ratio create a lot of possibilities in cookie production (Chevallier, Colonna, Della Valle, Lourdin, 2000; Chevallier, Della Valle, Colonna, Broyart, Trystram, 2002). In the latest years, research focus is oriented towards the improvement of textural and sensory properties of gluten-free cookies, as well as to their nutritionally rich formulations. According to the available data, when buckwheat flour has been used in a new gluten-free formulation the following levels of substitution in a gluten-free flour mixture were studied: 10% (Schober, O'Brien, McCarthy, Darnedde, Arendt, 2003), 20% (Sakač, Pestorić, Mišan, Nedeljković, Jambrec, Jovanov, Banjac, Torbica, Hadnađev, Mandić, 2015), 40% (Yamsaengsung, Berghofer, Schoenlechner, 2012), and 50% (Gambuś, Gambuś, Pastuszka, Wrona, Ziobro, Sabat, Mickowska, Nowotna, Sikora, 2009). Also, the effect of transglutaminase and canola proteins on quality characteristics of buckwheat gluten-free cookies was reported (Altındağ, Certel, Erem, Konak, 2015; Gerzhova, Mondor, Benali, Aider, 2016).

Rice flour is recommended as a safe ingredient in gluten-free diet, and is characterized by mild taste, it is colourless and has easily digestible carbohydrates. Substitution of rice with buckwheat flour was studied at the levels of 10, 20 and 30% by Torbica, Hadnađev, and Dapčević Hadnađev (2012) and Sakač, Pestorić, Mišan, Nedeljković, Jambrec, Jovanov, Banjac, Torbica, Hadnađev, and Mandić (2015). Based on the sensory properties of gluten-free rice and buckwheat cookies the formulation with 20% of buckwheat flour was found to have the most acceptable sensory properties and significantly improved nutritional profile, although the antioxidant capacity and mineral content were higher when 30% of buckwheat flour was used. The formulation of gluten-free rice and buckwheat cookies at the level of substitution 20% was as follows: 240 g of rice flour, 60 g of light buckwheat

flour, 75 g of water, 85 g of vegetable fat, 70 g of granulated sugar, 45 g of honey, 9 g of $NaHCO_3$, 9 g of diacetyl tartaric acid ester of monoglycerides, 4.5 g of carboxymethyl cellulose, and 2.1 g of salt (Torbica, Hadnađev, Dapčević Hadnađev, 2012). The substitution with light buckwheat flour enhanced antioxidant capacity, mineral content, protein and dietary fibre content of cookies (Tables 6, 7 and 8).

Mixtures of bean and pseudocereal flours including amaranth, kinoa and buckwheat in different ratios (25–100%) have been used in production of cookies (Schonlechner, Linsberger, Kaczyk, Berghofer, 2006). According to the above mentioned authors, amaranth and buckwheat cookies had better sensory characteristics than kinoa cookies, and cookies containing buckwheat flour were the crunchiest among tested cookies.

Schober, O'Brien, McCarthy, Darnedde, Arendt (2003) compared three cookie formulations with commercially available gluten-free and wheat flour cookies. Ingredients for the cookie formulation were as follows: 100 g of gluten-free mixture, 35 g of sugar, 30 g of palm oil, 27.5 g of eggs, 5 g of syrup, 2.5 g of guar gum, 0.5 g of salt, 0.5 g of sodium stearoil-2-lactilate, and 0.5 g of baking powder. The gluten-free flour mixture is given in Figure 2.

Table 6. Proximate composition of cookies (*Sakač, Pestorić, Mišan, Nedeljković, Jambrec, Jovanov, Banjac, Torbica, Hadnađev, Mandić, 2015*)

Component* (g/100 g)	Control sample	10% RF/LBF cookies	20% RF/LBF cookies	30% RF/LBF cookies
Protein	4.41 ± 0.13^{a}	4.48 ± 0.02^{ab}	4.60 ± 0.06^{b}	4.86 ± 0.12^{c}
Fat	19.6 ± 0.05^{a}	19.6 ± 0.05^{a}	19.8 ± 0.24^{a}	19.9 ± 0.30^{a}
Starch	53.2 ± 0.51^{a}	52.6 ± 0.74^{a}	52.3 ± 0.10^{a}	52.3 ± 0.04^{a}
Reducing sugars	15.3 ± 0.12^{c}	15.1 ± 0.09^{ab}	15.2 ± 0.05^{b}	15.0 ± 0.03^{a}
Ash	2.32 ± 0.02^{a}	2.41 ± 0.02^{b}	2.41 ± 0.02^{b}	2.46 ± 0.01^{c}
Total dietary fibre	1.93 ± 0.03^{a}	2.26 ± 0.09^{b}	2.55 ± 0.05^{c}	2.94 ± 0.04^{d}

* Results are presented on dry mass basis. Values are means of three determinations ± standard deviation. Values of the same row with the same superscript are not statistically different ($P < 0.05$). Control sample – RF cookies; 10% RF/LBF cookies – RF cookies enriched with LBF at the level of 10%; 20% RF/LBF cookies – RF cookies enriched with LBF at the level of 20%; 30% RF/LBF cookies – RF cookies enriched with LBF at the level of 30%; RF – rice flour; LBF – light buckwheat flour.

Table 7. Mineral content in rice cookies (RF cookies) and rice-buckwheat gluten-free cookies (10% RF/LBF cookies, 20% RF/LBF cookies and 30% RF/LBF cookies) (*Sakač, Pestorić, Mišan, Nedeljković, Jambrec, Jovanov, Banjac, Torbica, Hadnađev, Mandić, 2015*)

Material	Mineral (mg/kg)*					
	Mg	K	Zn	Fe	Mn	Cu
Control sample	382 ± 0.26[a]	823 ± 1.52[a]	7.83±0.02[a]	15.3 ± 0.20[a]	4.77 ± 0.44[a]	1.63 ± 0.10[a]
10% RF/LBF cookies	463 ± 10.7[b]	884 ± 5.23[b]	8.06±0.11[ab]	16.2 ± 0.33[b]	4.66 ± 0.12[a]	1.64 ± 0.07[a]
20% RF/LBF cookies	473 ± 36.7[b]	961 ± 1.92[c]	8.02±0.07[ab]	17.5 ± 0.11[c]	4.48 ± 0.03[a]	1.81 ± 0.05[b]
30% RF/LBF cookies	537 ± 2.94[c]	1063±27.42[d]	8.08±0.20[bc]	20.6 ± 0.79[d]	4.68 ± 0.14[a]	1.90 ± 0.07[b]

* Results are presented on dry mass basis.

Table 8. Phenolic content and antioxidant activities of rice cookies (RF cookies) and rice-buckwheat gluten-free cookies (10% RF/LBF cookies, 20% RF/LBF cookies and 30% RF/LBF cookies) (*Sakač, Pestorić, Mišan, Nedeljković, Jambrec, Jovanov, Banjac, Torbica, Hadnađev, Mandić, 2015*)

Extracts	Total phenolic content* (μg GAE/g)	Rutin* (μg/g)	AOA, IC_{50} (mg/mL)	Reducing activity, IC_{50} (mg/mL)	Scavenging activity on DPPH·, IC_{50} (mg/mL)	Chelating activity on Fe^{2+}, IC_{50} (mg/mL)
Control sample	955±46.2[a]	n.d.	25.0±0.21[a]	37.7±0.97[a]	23.2±0.69[a]	17.6±0.65[a]
10% RF/LBF cookies	1038±43.9[b]	25.1±0.54[a]	21.9±2.59[b]	32.3±0.63[b]	22.9±1.37[a]	4.79±0.73[b]
20% RF/LBF cookies	1248±13.7[c]	33.3±0.23[b]	18.7±1.70[c]	31.3±0.77[b]	16.8±0.75[b]	4.76±1.01[b]
30% RF/LBF cookies	1349±16.9[d]	40.1±0.58[c]	11.4±0.97[d]	29.0±1.15[c]	14.7±0.93[c]	4.64±0.52[b]

* Results are presented on dry mass basis.

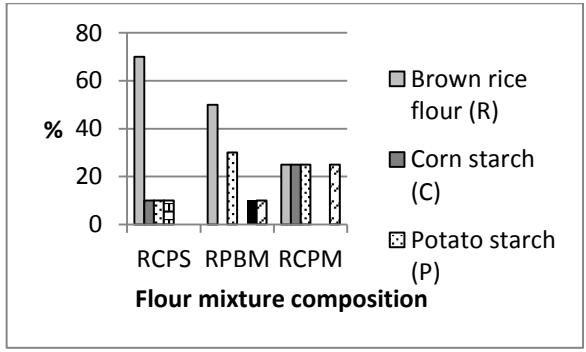

Figure 2. Gluten-free flour mixture composition used in formulations published by Schober, O'Brien, McCarthy, Darnedde, and Arendt (2003).

Commercially available gluten-free cookies were made of wheat starch, milk powder, modified corn starch, soy flour, glucose, salt and methyl hydroxyl propyl cellulose. One of three gluten-free formulations contained 10% of buckwheat flour in the flour mixture (Schober, O'Brien, McCarthy, Darnedde, Arendt, 2003). Water activity (a_w), water content, hardness, dimensions (diameter, spread ratio, thickness) and surface brightness (L*) of cookies, and dough hardness and stickiness were tested and a series of conclusions were derived from the obtained results. Hard and slightly sticky dough results in firm, thin and rounded cookies, while soft and sticky dough results in soft, thick and rounded cookies. Commercially available gluten-free cookies were characterized with high starch and low protein content, resulting in a low structure stability and high stickiness of such dough. Weak and sticky dough sticks to laminator rollers, dominantly stretches in one direction during lamination process and results in oval cookies. Cookies containing buckwheat flour possessed highest a_w value and water content, and they had the darkest surface, as a consequence of the presence of buckwheat flour.

Another formulation of gluten-free cookies was given and studied by Gambuś, Gambuś, Pastuszka, Wrona, Ziobro, Sabat, Mickowska, Nowotna, and Sikora (2009). The authors used the following recipe: gluten-free flour mixture (260 g), pectin (10 g), margarine (150 g), gluten-free baking powder (13 g), eggs (45 g), sucrose (120 g), and vanillin (2 g). The used gluten-free flour mixture is given in Figure 3.

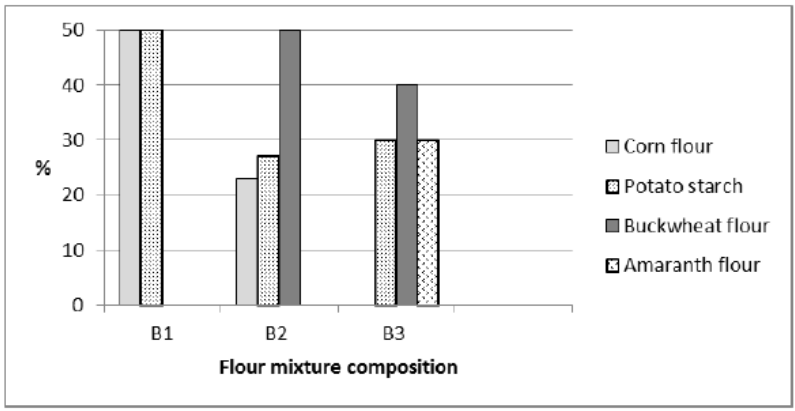

Figure 3. Gluten-free flour mixture used in formulations published by Gambuś, Gambuś, Pastuszka, Wrona, Ziobro, Sabat, Mickowska, Nowotna, and Sikora (2009).

Sensory evaluation showed that B3-cookies were the best with the total score of 4.83. B3-cookies did not contain corn flour, it was substituted with the mixture of buckwheat flour (40%) and amaranth flour (30%). B2-cookies, contained 50% of buckwheat flour, gained lower score of 4.46 due to a intense odour and taste of buckwheat flour. Hardness of cookies was measured during 30 days of storage. Significantly lower hardness was obtained for gluten-free cookies which did not contain flour from pseudocereals, B1, in comparison to pseudocereal-containing cookies, B2 and B3, measured from 1st to 30st day of storage. On the day of baking the cookies containing buckwheat and amaranth flour (B3) were less hard due to the starch structure of amaranth. Namely, amylopectin is the dominant component in starch from amaranth, with lower tendency for starch retrogradation in comparison with the starches of other botanical origin. Lower retrogradation causes lower hardness of cookies on the day when they were baked.

Nutritional profile was significantly improved when pseudocereals were present in the formulation. Three-fold higher protein content was recorded in B3-cookies in comparison with B1-cookies. Also, the level of dietary fibre was significantly higher when buckwheat and amaranth flour (B2 and B3) were used in comparison to the control (B1). Formulations including pseudocereals resulted in cookies with the improvement of amino acid composition of created cookies. Content of essential amino acids was 2.5 times higher in B3 and 30% higher in B2-cookies, when compared to the

control (B1-cookies). Content of macro- and microminerals was higher in B2 and B3-cookies than in B1-cookies and it is considered to be a significant improvement, especially knowing the fact that the diet of coeliac patients is usually low in calcium, iron, zinc and selenium (Kupper, 2005).

Influence of the addition of chickpea in different types of buckwheat cookies was studied by Yamsaengsung, Berghofer, and Schoenlechner (2012). Buckwheat cookie recipe was as follows: 150 g of buckwheat flour which has been substituted with chickpea flour within the range 0–100%, 62.5 g of vegetable fat, 62.5 g of sugar, 60 g of eggs, 4.5 g of guar gum, 1.5 g of baking powder, 1 g of salt, and 1 g of vanillin sugar. The addition of chickpea significantly increased the yellowness of cookies, and the maximum rate was obtained at the level of 40% according to the sensory panel results. Chickpea reduced the spread factor of buckwheat cookies, and their hardness was increased with the addition of chickpea flour. Sensory evaluation demonstrated that the addition of chickpea could increase the acceptability of cookies when it was added at the level of 60% in the cookie formulation. The authors concluded that the high protein chickpea flour improved nutritional profile and increased the consumer acceptability of cookies.

Transglutaminase is an enzyme used in food industry and can modify protein functionality and promote protein cross linking, thus modifying the viscoelastic properties of dough. Altındağ, Certel, Erem, and Konak (2015) studied the influence of transglutaminase addition on quality characteristics of gluten-free cookies made of buckwheat flour (100%), mixtures buckwheat: corn (50:50%), buckwheat:rice (50:50%), and buckwheat:corn:rice (50:25: 25%). Cookie recipe was: 1000 g of flour, 420 g of sugar, 400 g of vegetable margarine, 10 g of skimmed milk powder, 12.5 g of salt, 5 g of ammonium bicarbonate, 10 g of sodium bicarbonate, 15 g of high fructose corn syrup, and 220 g of distilled water. All cookies were also prepared with the addition of 0.002% transglutaminase based on flour weight. Thickness and the spread ratio were affected by flour type and enzyme addition. Moisture and spread ratio were higher in cookies with transglutaminase addition in comparison to cookies without enzyme addition. Observed decrease in hardness could contribute to an increase in moisture of cookies prepared with the addition of transglutaminase as a consequence of changes in protein structure. Also, it could be seen that cookies prepared with the buckwheat flour had the highest hardness value in comparison to other cookies.

Influence of isolates and concentrates of canola proteins on the quality parameters of rice buckwheat gluten-free cookies were also studied (Gerzhova, Mondor, Benali, Aider, 2016). To a slightly modified recipe of Torbica,

Hadnađev, and Dapčević Hadnađev (2012), the part of rice flour was substituted with canola proteins at the levels of 3, 6 and 9%. Formulation was as follows: 60 g of buckwheat flour, 240 g of rice flour, 75 g of sugar, 100 g of margarine, 15 g of honey, 3 g of sodium bicarbonate, 2.1 g of salt, and 105 g of water. Spread ratio decreased for all samples and the increase in diameter and thickness was recorded when the samples compared to the control cookies. The addition of canola isolate at all investigated levels and the addition of 3% of concentrate significantly decreased the hardness of cookies, but this parameter was increased when concentrate was added at the levels of 6 and 9%. Overall sensory acceptability of cookies was improved with the addition of canola proteins.

Gums are widely used in food industry, since they positively affect quality characteristics of food. Kaur, Sandhu, Arora, and Sharma (2015) studied how addition of various gums influence physicochemical and sensory characteristics of buckwheat gluten-free cookies. Gum acacia, guar gum, gum tragacanth and xanthan gum were used in cookie formulations. Cookies with gums had higher moisture content, thickness, diameter and were softer than cookies without gums. Sensory properties were significantly improved with gum addition, where among all gums xanthane gave the best results.

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Professional Appointments:

2015-present, Principal Research Fellow
2009-2015, Research Associate
2000-2009, Research Assistant
1997-2000, Teaching Assistant

Honors:

2011 – Kraft Foods Europe Award for Cereal Research for the Best presentation at 11th European Nutrition Conference-FENS, Madrid, Spain.

2011 – The best abstract award at 4th International Congress on Food and Nutrition together with the 3rd SAFE Consortium International Congress on Food Safety, Istanbul, Turkey.

Publications Last 3 Years:

[1] Mišan, A., Sakač, M., Medić, Đ., Tadić, V., Marković, G., Gyura, J., Pagano, E., Izzo, A., Borrelli, F., Šarić, B., Milovanović, I., Milić, N. (2016). Antioxidant and physicochemical properties of hydrogen peroxide treated sugar beet dietary fibre. Phytotherapy Research, 30, 855-860.

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[15] Mandić, A., Sedej, I., Sakač, M., Mišan, A. (2013). Static headspace gas chromatographic method for aldehyde determination in crackers. Food Analytical Methods, 6, 1, 61-68.

[16] Šimurina, O., Sakač, M., Sedej, I., Psodorov, Đ., Mandić, A., Mišan, A., Pestorić, M., Tadić, V., Đurendić-Brenesel, M., Pilija, V., Milić, M., Popović, T., Arsić, A., Arsić, I.: Bread with Flaxseed and Plant Mixture for Metabolism Improvement and Regulation of Body Weight. Patent Reg. No. 52819, 01.11.2013 (Application No. P-2009/0141, The Intellectual Property Office of Republic of Serbia).

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Chapter 3

GLUTEN-FREE DIET AND GLUTEN-RELATED DISORDERS

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ABSTRACT

Celiac disease (CD) is an autoimmune disorder, occurring in genetically predisposed individuals, triggered by the ingestion of dietary gluten, the major protein component in wheat and other related cereals. In many areas of the world, CD is one of the commonest lifelong disorders affecting around 1% of the population. Indeed, CD research is changing rapidly as gluten-related disorders have gradually emerged as an epidemiologically relevant phenomenon with a global prevalence. Among such disorders, CD and wheat allergy (WA) have been extensively studied although they are not the only entities, as non-celiac gluten sensitivity (NCGS) has been recently re-discovered and appears to be a very common disorder, in particular in the U.S.A.

A lifelong gluten-free diet (GFD) is currently the only available treatment for such disorders. Clinical manifestations associated with untreated patients, including intestinal but also extra-intestinal manifestations are ameliorated with a GFD. However, despite a strict

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adherence to the diet is essential to reduce symptoms, estimated compliance rates vary considerably in CD (17-80%). Despite the importance of monitoring the GFD, there are no clear guidelines for assessing the outcome or for exploring its adherence. Available methods are insufficiently sensitive to detect occasional dietary transgressions that may cause gut mucosal damage. Thus, detection of gluten immunogenic peptides (GIP) in feces and urine has been proposed as new non-invasive biomarker to determine gluten intake and monitor GFD compliance in patients with CD. This method has showed high sensibility and significant correlation with consumed gluten, enabling assessment of gluten exposure early after ingestion and could aid in the diagnosis and clinical management of non-responsive CD and refractory CD.

Keywords: celiac disease, gluten immunogenic peptides, gluten-free diet, non-celiac gluten sensitivity, wheat allergy

INTRODUCTION

Wheat, rice and maize are the most widely consumed food grains in the world. Wheat, the most extensively grown crop, because of its nutritional goodness, and the ability of its flour to produce a variety of tasty and satisfying foods. This is consequence of the unique viscoelastic properties of wheat doughs, which allow entrapping CO_2 during fermentation, enabling the preparation of leavened breads and other baked products. The wheat products make substantial contributions to the dietary intake of energy and protein, and supply dietary fiber, minerals, vitamins, and phytochemicals (Kasarda, 2013; Lundin, 2014). However, wheat products also have negative impacts on human health, in relation to allergies and intolerances. These disorders are related to gluten exposure, the main structural protein complex of wheat and other cereals as rye, barley and oats. Gluten is a complex mixture of proteins called prolamins. This protein fraction has specific name: wheat prolamins are termed gliadins and glutenins, barley prolamins are hordeins, rye prolamins are secalin and those from oats are avenins. A common characteristic of these proteins is the presence of multiple proline and glutamine residues, making them resistant to gastrointestinal digestion and more exposed to deamination by tissue transglutaminase.

Different pathologies are associated with cereal intake, which appear to be increasing in importance: a) CD, which is an intolerance to gluten not only from wheat, but also rye, barley and some oats, and affects both children and

adults throughout the world at various frequencies (Trier, 1998; Sollid, 2002; Kagnoff, 2007; Ludvigsson et al., 2013), b) food allergy to wheat proteins, which affects 0.2-0.5% of the population (Zuidmeer et al., 2008), and c) NCGS, a pathology in which gluten ingestion leads to morphological or symptomatic manifestations despite the absence of CD and WA (Sapone et al., 2012), with highly variable prevalence from 0.6 to 6% (Volta et al., 2015).

GLUTEN-RELATED DISORDERS

Celiac Disease

Celiac disease is an autoimmune disorder developed in genetically (HLADQ2/8) predisposed individuals and caused by a permanent intolerance to gluten contained in some cereals, such wheat, rye, barley and some oat varieties that leads to a chronic inflammation of the small intestine (Jabri and Sollid, 2006; Makharia et al., 2012; Ludvigsson et al., 2013) (Figure 1).

The most accepted model for explaining CD immunopathogenesis is the two-signal model (Brandtzaeg, 2006) characterized by a first innate immune response followed by a secondary antigen-specific adaptive response. Some peptides like the 19-mer gliadin peptide trigger an innate immune response (Maiuri et al., 2003) mainly characterized by the production of IL-15 by epithelial cells. The result is the disruption of the epithelial barrier, by increasing the permeability and inducing enterocyte apoptosis (Maiuri et al., 2000). As a consequence, immuno-adaptive peptides, like the 33-mer gliadin peptide, can now reach the lamina propria where they are deaminated by the enzyme tissue transglutaminase. Such deamidation provides a negative load to gliadin peptides and hence enhancing their affinity to fit in the HLA-DQ2/8 bound, which is also the “susceptibility gene” in CD, expressed on the surface of dendritic cells (Qiao et al., 2004; Ráki et al., 2006; Tollefsen et al., 2006). Dendritic cells promote differentiation of pro-inflammatory antigen-specific effector T-cell (Stagg et al., 2002; Hart et al., 2004) driving progression of the pro-inflammatory antigen-specific adaptive immune response, which will turn into the symptomatology of the disease.

In the past, most patients diagnosed with CD were children with severe organic manifestations, but in more recent years there has been an increase in diagnosis of adults and pauci-symptomatic patients (Ludvigsson et al., 2013). The clinical manifestations of CD are heterogeneous and range from the so-called “classical” syndrome with diarrhea, steatorrhea,

weight loss, malnutrition, bloating, flatulence, abdominal pain and selective malabsorption of micronutrients (iron, vitamin B_{12}, calcium). Non-classical features include irritable-bowel-type symptoms, hypertransaminasemia, growth retardation, iron deficiency anemia, weight loss, chronic fatigue, delayed puberty, amenorrhea, cerebellar ataxia, peripheral neuropathy, etc. (Rubio-Tapia et al., 2013). CD can be associated with other disorders, such as autoimmune diseases (type-I diabetes, autoimmune thyroiditis, autoimmune hepatitis, etc.), infertility and dermatitis herpetiformis (Rubio-Tapia and Murray, 2010). Children and adolescents with Down's syndrome, Turner's syndrome, Williams syndrome, IgA deficiency, autoimmune thyroiditis, type-1 diabetes, or autoimmune disorders of the liver or first-degree relatives of celiac patients indicate an increased risk of CD (Husby et al., 2012; Rubio-Tapia et al., 2013). The history of CD can be rarely complicated by refractory celiac disease or malignancies including lymphoproliferative disorders and carcinoma of the small bowel (Rostami et al., 2001; Freeman, 2004; Catassi et al., 2005; Bardella et al., 2009; Elli et al., 2012; Elli et al., 2015; Woodward, 2016).

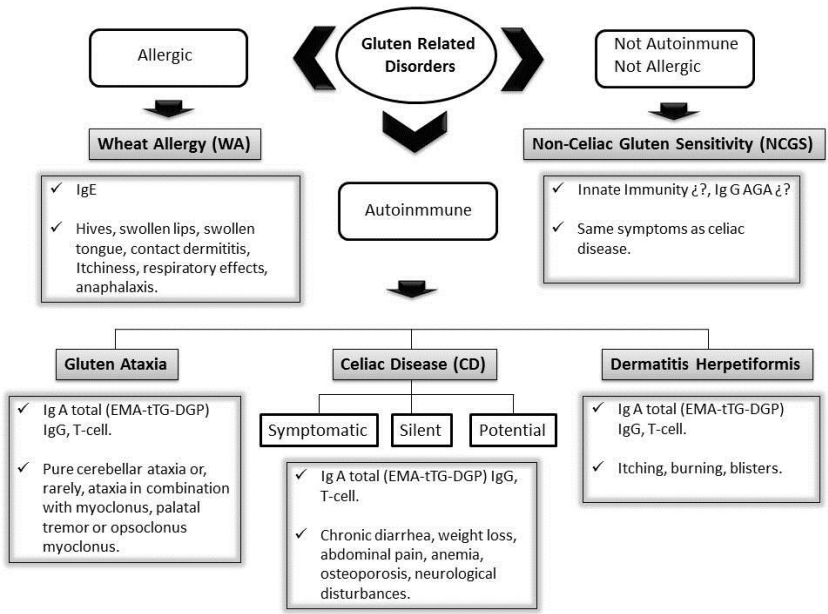

Figure 1. Classification of gluten-related disorders (modified according Sapone et al., 2012).

The diagnosis of CD is base on a combination of findings from patient's clinical history, genetic markers (HLA-DQ2/DQ8), specific serological antibodies, and histopathological analyses of the duodenal specimens. The preferred serologic test for the detection of CD in subjects above 2 years of age is the anti-transglutaminase IgA antibody (TTG) (van der Windt et al., 2010). The anti-endomysium antibody (EMA) can be used as a confirmatory test in cases of uncertain diagnosis in high-risk populations (Leffer and Schuppan, 2010). However, EMA test is expensive (monkey esophagus or human umbilical cord are used as substrates) and operator dependent due to the interpretation of immunofluorescent pattern. Deamidated gliadin peptide (DGP) IgA and IgG, substituting the anti-gliadin antibodies, are used in combination with TTG IgA in children who are less than 2 years old (Husby et al., 2012).

The histopathological analyses are usually based on the Marsh-Oberhuber histological classification which ranges from a normal mucosa (marsh 0) to the appearance of lymphocytic infiltration (marsh 1), crypt hyperplasia (marsh 2), and different levels of villous atrophy (marsh 3a-c), although this classification is still subjective (Peña, 2015). More objective and practical classifications have been proposed in the last years such as by Corazza and Villanacci (2005) and by Ensari (2010).

Wheat Allergy

WA is defined as an adverse immunologic reaction to wheat proteins (Figure 1). Depending on the route of allergen exposure, WA is classified into occupational asthma (baker's asthma) and rhinitis; food allergy (FA), affecting the skin, the gastrointestinal tract or the respiratory tract; wheat dependent exercise-induced anaphylaxis (WDEIA) and contact urticaria. IgE antibodies play a central role in the pathogenesis of these diseases.

Many allergenic proteins are involved in WA (Elli et al., 2015) and the latest updated version of the WHO/IUIS Allergen Nomenclature Database describes 24 different well classified wheat allergens (http://www.allergen.org/search.php?Species=Triticum%20aestivum). There are many of those responsible for different clinical conditions and other specifically associated with FA (non specific lipid transfer protein and gliadins), respiratory symptoms (alpha-amylase/trypsin inhibitor), WDEIA (omega-5 gliadin), or contact urticaria (HMW glutenins) (Tatham and Shewry, 2008; Inomata, 2009).

These allergies can affect in both children and adults. The majority of wheat allergic children suffer from moderate-to-severe atopic dermatitis and wheat ingestion may elicit typical IgE mediated reactions, including urticaria, angioedema, bronchial obstruction, nausea and abdominal pain, or in severe cases systemic anaphylaxis. Commonly WA in children is out growed by school-age (Ramesh, 2008; Keet et al., 2009). In adults FA to ingested wheat is infrequent and difficult to recognize, the most frequent symptoms are diarrea and bloating. The most common variant in adults is WDEIA, where symptoms result from the combination of causative food intake and physical exercise (as well as non-steroidal anti-inflammatory drugs or alcohol). Baker's asthma and rinitis are well characterized allergic responses to the inhalation of wheat and cereal flours and dusts. Moreover, Baker's asthma is recognized as one of the most common types of occupational asthma (Tatham and Shewry, 2008).

Skin prick tests and in vitro IgE assays are first-level diagnostics for WA. However, the positive predictive value of these tests is less than 75%, particularly in adults due to the cross-reactivity with grass pollens. Therefore, challenge tests remain the gold standard for WA diagnosis, but they are cumbersome and potentially dangerous (Sapone et al., 2012).

Non-Celiac Gluten Sensitivity

In addition to CD and WA, there is a third gluten related syndrome where the immunological mechanisms are not related to the presence of IgE, like in WA, or an adaptive immune response characterized by the presence of gluten-reactive T-cells and antibodies directed against TTG or deaminated gluten peptides like in CD (Figure 1). This syndrome, NCGS, is thought to arise from an innate immune response to dietary gluten not coupled to a secondary adaptive immune response (Escudero-Hernández et al., 2016). NCGS occurs more commonly in young women (3rd-4th decade of life), while it is rare in childhood. The prevalence is extremely variable ranging from 0.6% in primary care to 6% in referral centers (Volta et al., 2015).

NCGS is still a poorly defined syndrome, characterized by gastrointestinal such as abdominal pain, bloating and altered bowel habit, and extraintestinal symptoms such as fatigue, headache, bone or joint pain, mood disorders and skin manifestations (e.g., eczema or rash) elicited by gluten ingestion in patients without CD and WA (Catassi et al., 2013). Symptoms disappear quickly in a few hours or days after gluten removal from the diet and recur

rapidly when gluten is reintroduced. In fact, some individuals experience distress when eating gluten-containing products and show improvement when following a GFD may have NCGS instead of CD (Sapone et al., 2010 and 2012). The small intestine of NCGS patients is usually normal. However, the two conditions cannot be distinguished clinically, since the symptoms experienced by NCGS patients are often seen in CD. However, no genetic marker has been identified in NCGS; in particular, no correlation with HLA-DQ2 and -DQ8, markers of CD, has been demonstrated.

In addition to gluten, other triggers, including amylase-trypsin inhibitors (ATIs) and fermentable oligo-di-monosaccharides and polyols (FODMAPs), can be involved in determining NCGS (Biesiekierski et al., 2013; Zanini et al., 2015). However, the relative contribution exerted by single dietary factors in NCGS-related symptoms is still unclear because cereals contain both ATIs and FODMAPs admixed with gluten.

So far, there are no available biomarkers for NCGS, the diagnosis of NCGS is based on the clinical response to GFD and the exclusion of other syndromes as WA (presence of IgE) and CD (presence of TTG antibodies). Nevertheless, about 50% of patients are positive for IgG AGA, which disappear quickly after GFD together with symptoms. Double-blind, placebo-controlled, cross-over trial is the best procedure to confirm the diagnosis of NCGS.

GLUTEN-FREE DIET

In general, treatment of gluten-related disorders is based on excluding gluten-containing cereals from the diet. Wheat, barley and rye proteins are completely excluded in a GFD. The cereals are replaced by gluten-free cereals such as rice, corn, buckwheat and millet. Some leguminosae such as quinoa, amaranth and soybean are particularly useful due to their high protein content and quality as replacements for their gluten containing analogues. Moreover, non-processed food as meat, fish, poultry, egg, fruit and vegetables are recommended to facilitate GFD adherence and secure the nutritional value of the diet.

Gluten and wheat are often interchanged and confused. A food labelled “wheat-free” may be safe for someone with WA but not necessarily for someone with NCGS or CD. For example, a person with a WA may tolerate bread labelled “wheat-free” but it may contain other grains such as barley, rye, or oat, which a person with NCGS or CD may not tolerate. Identification and

labelling of gluten-free foods is a significant issue in adhering to a GFD (Capili et al., 2014).

In CD, the goal of this treatment is to relieve symptoms, achieve mucosal healing and avoid complications of the disease (Ludvigsson et al., 2014). However, although adhering to a GFD might seem simple, to abstain from the abundance of gluten-containing food in the Western diet can be challenging and the treatment can considerably affect the patient's quality of life (Vriezinga et al., 2015). Estimated compliance rates vary considerably (17-80%), depending on factors such as the patient's age or the age at diagnosis of CD, among others (Ciacci et al. 2002; Högberg et al., 2003; Pietzak 2005; Herman et al., 2012). Thereby, there is extensive research on GFD and how this may influence the clinical course. The poor dietary adherence has shown to be negative to promote other autoimmune disease (Ventura et al., 1999; Corrao et al. 2001), fertility problems (Rampertab et al., 2003; Ludvigsson, et al., 2005; Khashan et al., 2010), and increased risk of bone fracture (Lebowhl et al., 2013) or lymphoma (Silano et al. 2008; Olén et al. 2011). In addition, after adoption of the GFD, 4-30% of patients with CD reported persisting symptoms and are considered to be affected by nonresponsive CD (NRCD) (Ludvigsson et al., 2014). However, only 10% of these NRCD patients have refractory CD, being inadvertent or deliberate gluten exposure the most frequent cause of NRCD (Leffler et al., 2007).

Most patients with suspicion of NCGS are referred to the dietitian and a GFD is instituted. In NCGS, the health benefit from GFD seems to be mostly symptomatic, and so the degree of diet strictness is defined by individual tolerance. However, there are several unresolved issues in the dietary management of NCGS. Compared to CD patients, nutritional status at diagnosis and follow-up is not well described in NCGS patients. Moreover, to our knowledge, there is no information on GFD adherence in these patients. Another important issue to consider is the effect of ATIs from gluten-containing cereals in this disorder. Certains studies have suggested that ATIs may signal through TLR4 in monocytes, macrophages and DC from the intestinal mucosa and act as immune adjuvants for gluten in biopsy cultures. However, as a GFD is an ATI-free diet, it has been suggested that patients with ATI sensitivity could follow a GFD (Schuppan et al., 2015). In addition, the GFD is a healthy diet as it avoids the consumption of processed and manufactured foods hence favouring the intake of fresh fruit and vegetables and sauce-free grilled products. Therefore, the acquisition of a healthy lifestyle and diet could also be behind, at least to some extent, of the clinical

improvement and increased well-being that several individuals have reported following the introduction of the GFD (Escudero-Hernández et al., 2016).

Monitoring of Gluten-Free Diet Compliance

Despite the importance of monitoring the GFD, there are no clear guidelines for assessing the outcome or for exploring its adherence. In addition, there is no consensus regarding the frequency of monitoring or the best measurements for assessing compliance and outcome (Bai et al., 2013). A variety of surrogate markers are available to assess the GFD compliance including patient self-report about level of adherence, dietary history, an evaluation conducted by a professional nutritionist, small-bowel biopsy, and serologic screening tests Nevertheless, the lack of a standardized and accurate measure of GFD adherence is a significant problem both clinically and in research.

Self-report is a problematic method for measuring adherence to the GFD since individuals tend to inaccurately report their level of adherence, whether intentionally or unintentionally. Similarly, dietary interviews, although shown to be helpful in the determination of diet compliance, are often not possible to standardize, are subjective or rely on a truthful response from the patient, and cannot identify involuntary infringements.

The use of endoscopies to collect biopsies and assess mucosal healing is the gold standard; however, it is invasive, expensive and not a practical method for serial monitoring. Therefore, there is no consensus on the role of follow-up biopsies, especially in asymptomatic patients in whom clinical improvement is seen (Pietzak, 2005; Husby et al., 2012).

Many physicians rely on serology at follow-up. Unfortunately, data clearly shows that serology at follow-up has a poor correlation with mucosal healing, and reliance on serology may therefore underestimate the activity of CD (Dickey et al., 2000; Kaukinen et al., 2002; Vahedi et al., 2003; Kaukinen et al. 2007). CD-especific antibodies as TTG or DGP have been proposed as markers of GFD compliance. However, IgA- and IgG-class tests usually can take 6 to 24 months to decrease after the antigen source has been eliminated from the diet. Hence, serology does not predict recovery and is not useful for follow-up. In NCGS, AGA of the IgG class have been suggested as a possible marker of GFD compliance, since these antibodies disappear in a few months together with symptom resolution. Nevertheless, these antibodies appear only

positive in 50% of diagnosed patients with NCGS (Caio et al., 2014; Volta et al., 2015).

Other tests suggested as suitable diet monitoring markers were the permeability test, fecal calprotectine or REG Iα (Duerksen et al., 2005; Ertekin et al., 2010; Planas et al., 2011), but, they are not specific for CD and can appear in other gastrointestinal diseases.

Since the above tests to monitoring GFD compliance only measure the consequences of dietary transgressions, there is a need for accurate, non-invasive tools for managing patients on the way to show gluten intake and avoid the harmful aftermaths in addition to assess the efficacy of new drugs or non-dietary therapies in such patients on an ongoing basis.

Detection of Gluten Immunogenic Peptides

CD is triggered by the presence of peptides, from the fragmentation of gluten, that are not digested by human proteases and that are toxic for CD patients. Shan et al. (2002) have shown by *in vitro* and *in vivo* studies in rats and humans that a 33-mer peptide from α2-gliadin is stable toward breakdown by all gastric, pancreatic, and intestinal brush border membrane endoproteases. This peptide was identified as the primary initiator of the inflammatory response to gluten in celiac patients (Shan et al., 2002). Toward the assessment of food toxicity, G12 and A1 monoclonal antibodies were obtained against 33-mer peptides. These antibodies have proved to be very useful for the detection of toxic peptides in food samples and for the enzymatic detoxification of gluten in clinical research (Morón et al., 2008a and 2008b). In addition, anti-33-mer G12-based immunoassays showed that >30% of the gliadin-reactive peptides remained intact after hydrolysis during *in vitro* simulated gastrointestinal digestion (Comino et al., 2012). Based on these findings, Comino et al., (2012) described a novel method to monitor the GFD by detection of GIP in human feces on the basis of the use of the anti-gliadin 33-mer G12 antibody. The resistance of gluten peptides to gastrointestinal digestion, in particular peptides related to the immunotoxic 33-mer peptide, ensures that a significant part of the ingested gluten peptides are excreted in feces. Consequently, the recovery of measurable amounts of the immunotoxic fraction in feces indicate that gluten has passed through the digestive tract and, therefore, that gluten has been consumed. GIP were detected in feces of healthy individuals after consumption of a normal gluten-containing diet, a GFD combined with controlled ingestion of a fixed amount of gluten (Comino et al., 2012). Subsequent studies have showed 29.8% of celiac patients in GFD displayed GIP levels in stools. There was significant association between age

and GIP in stools that revealed increasing dietary transgressions with advancing age (39.2% in subjects ≥13 years old) and with gender in certain age groups (60% in men ≥13 years old). No association was found between fecal GIP and dietary questionnaire or anti-tTG antibodies. Detection of gluten peptides in stools reveals limitations of traditional methods for monitoring GFD in celiac patients. Therefore, GIP-ELISA enables direct and quantitative assessment of gluten exposure early after ingestion and could aid in the diagnosis and clinical management of non-responsive CD and refractory CD (Comino et al., 2016).

GIP can also be detected in urine samples 6 to 48 h after gluten intake (Moreno et al., 2015), whereas it has been shown to remain detectable in stools for up to 4 days (Comino et al., 2012). Urine analysis could be used in conjunction with the fecal test for early detection of dietary infringements and the monitoring of GFD compliance in CD. Moreover, GIP are detected in urine samples after intake of 25 mg of gluten, which is below the minimum amount of gluten consumption known to cause histological abnormalities in CD patients (50 mg gluten/day) (Moreno et al., 2015) proving therefore their utility as non-invasive biomarkers (as opposed to a blood test) to monitor compliance to the GFD.

CONCLUSION

Different pathologies are associated with cereal intake, which appear to be increasing in importance. CD is a systemic autoimmune disorder triggered by gluten consumption in genetically susceptible individuals. It exhibits several clinical features such as blood auto-antibodies (TTG, DGP), plus variable degrees of damage in the small intestinal mucosa. However, it is now becoming apparent that reactions to gluten are not limited to CD, rather we now appreciate the existence of a spectrum of gluten-related disorders including WA and NCGS. NCGS has been recognized as a new gluten-related syndrome by the scientific community, but the knowledge on this syndrome is still incomplete with few certainties and many unsettled issues.

Treatment of gluten-related disorders is based on excluding gluten-containing cereals from the diet. Patients should be followed-up with dietary interviews and serology as CD markers to ensure adherence to the diet, but none of these methods offer an accurate measure of dietary compliance. Therefore, new tools based on the determination of GIP in feces and urine

have been developed for a better monitoring of compliance to the GFD in a less subjective and non-invasive manner.

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Chapter 4

THE POLEMIC ABOUT GLUTEN

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ABSTRACT

Gluten is a specific combination of protein that is present in wheat, rye, oats, barley, and their derivatives, such as flour, bread, cakes, and biscuits. This combination consists of two protein fractions called prolamins and glutenins. Prolamins are given different names according to their origin: gliadin in wheat, secalin in rye, and hordein in barley. Prolamins are the cause of the allergic reaction characteristic of celiac disease. Celiac disease is the result of genetically susceptible individuals allergic to gluten, causing inflammatory damage and abnormal intestinal mucosa. Therefore it is an autoimmune disease. Because of the effects that gluten causes in some patients, foods that contain gluten have been viewed with suspicion and wrongly removed from the diet, even when it comes to healthy individuals who have no sensitivity or allergy to gluten. The aim of this chapter is to explain and clarify doubts about gluten and its effects in the body, both in celiac patients and in those without the disease.

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Keywords: gluten-free diet, celiac disease, health effects, gluten sensitivity, wheat, bakery products

INTRODUCTION

Gluten is a protein complex present in many cereals and foods derived from oat base, wheat, rye, barley, and/or malt. Gluten consumption has generated a lot of controversy. This protein fraction has been seen as the enemy of health. In many cases, gluten has been taxed as a villain in the functioning of the body.

Wheat is the most important cereal used in baking. The proteins present in the cereal can be classified into prolamins (soluble in ethanol) and glutenins (insoluble in ethanol). These proteins when hydrated form a protein complex called gluten, which is responsible for the viscoelastic properties of dough, which are fundamental for the development of all kinds of bakery products (Preichardt and Gularte, 2013). Like other glutinous grains, wheat is a staple food in the diet for much of the world's population (Mansueto et al., 2014).

The prolamins, which are called in wheat gliadin, secalin in rye, and barley hordein, are responsible for celiac disease, a disease that causes autoimmune allergy in genetically susceptible individuals. The gluten-free diet has propagated far, not including celiac patients, with claims for slimming and a healthier diet, despite the little basement and insufficient medical evidence.

The propagation of gluten-free diet is due to the growing number of people who do not have celiac disease, but have gastrointestinal or nongastrointestinal symptoms alleviated after removal of gluten from the diet. This condition is called nonceliac gluten sensitivity (NCGS) (Catassi et al., 2013); some authors call this sensitivity to wheat, assuming the responsible for the symptoms may not be gluten (Carroccio, Rini, Mansueto, 2014). Since there is no knowledge about the agents responsible for the onset of symptoms in these cases, one must be careful in describing and using these names. Furthermore, exclusion of foods rich in nonabsorbable sugars and polyoils (FODMAPs) from the diet has also been shown to improve symptoms in people with NCGS (Biesiekierski et al., 2013), as happens with irritable bowel syndrome (IBS) (Verdu, Armstrong, Murray, 2009), which reinforces the idea that gluten is unrelated to these symptoms.

In this sense, the aim of this chapter is to explain and clarify doubts about gluten and its effects in the body, both in celiac patients and with some kinds of sensitivity to gluten, as in those without celiac disease.

1. BAKERY PRODUCTS

Bakery products, especially bread, are a very widespread food and consumed worldwide; therefore they are considered an important source of nutrients for the population, being a rich source of carbohydrates. Bakery products are present in the day-to-day lives of Brazilians and represent, according to the Brazilian Association of Bakery Industry, ABIP (2016), an annual turnover of R$84.7 billion.

The recommended amount per capita for bread according to the WHO is at least 60 kg per person per year; however, the Brazilian consumes 33.5 kg per person per year (Cunha, 2012).

Bakery products' main ingredient is wheat flour, with rheological characteristics attributed to gluten. The wide application of wheat flour in the preparation of bakery products are due to extensibility and viscoelasticity attributed to gluten proteins, assisting in retention of gas, enabling development of an adequate volume of products. These are properties that provide for baking flour characteristics, appropriate and specific to the texture of breads, cakes, and pastries (Fenema, 1996; Hoseney, 1994).

2. GLUTEN

Gluten is a specific combination of insoluble protein, known as prolamin (soluble in ethanol), and glutenin (insoluble in ethanol). The prolamins are given different names according to their origin: gliadin in wheat, secalin in rye, and hordein in barley. In wheat, gluten is formed by gliadin and glutenin (Cauvain and Young, 2009; Hoseney, 1994; Sgarbieri, 1996).

Gluten is a complex mixture of proteins with viscoelastic properties, containing a series of polypeptides; about half of the protein is monomeric (gliadin), and the remainder, polypeptide disulfide cross-linked to form the glutenin fraction of the polymer. The molecular weights (MW) of native proteins vary from about 30,000 to over 10 million (Wieser, 2007).

Gluten is of utmost importance for the food industry, because it provides high-quality characteristics, especially in bakery products. In the case of wheat flour, gliadin is responsible for dough cohesiveness, and glutenin is responsible for resistance to extension of the dough (Hoseney, 1994). The glutenin forms a network of fibers: gliadins globular are trapped (Day et al., 2006). When wheat flour is mixed with water and homogenized mechanically

in production of bakery products, there is hydration of the gliadin and glutenin proteins flour constructing a protein complex for formation of covalent and noncovalent bonds, resulting in gluten (Wrigley, Bek, Bushuk, 2006). This gives the dough unique viscoelastic properties and the ability to retain gases. Figure 1 is a representation of gluten's viscoelastic network and the place occupied by the gliadin and glutenin fractions within its structure.

The mass of gluten has rubbery appearance, being able to deform and stretch to recover shape and entrap gases. These characteristics are very important for the production of bread and all fermented products and many bakery products (Cauvain and Young, 2009). Due to its unique technological properties, compared with other proteins, gluten proteins are widely used not only in baked products, but also as an ingredient in a variety of food products (Day et al., 2006).

Among all the cereals, these properties are almost unique to wheat. However as already seen, some other cereal flours such as those derived from barley and rye can produce gluten, but in a lesser degree than normally observed in wheat flour (Cauvain and Young, 2009). Some individuals, however, have a genetic predisposition that causes the small intestine to present an inflammatory lesion due to gluten allergy, thus preventing the patient from consuming foods, beverages, and medications containing this substance (Sivaramakrishnan et al., 2004).

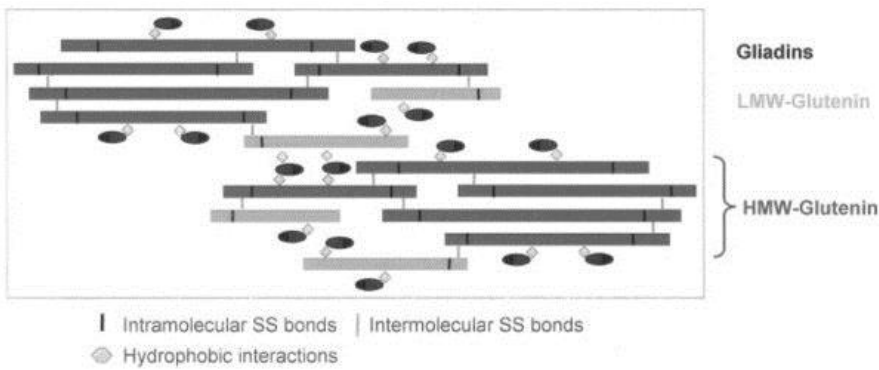

Figure 1. Representation of the gluten viscoelastic network and the place occupied by the gliadin and glutenin fractions within its structure. *Source:* Apper-Bossard et al. (2013).

3. GLUTEN HEALTH EFFECTS

Lately gluten has been seen as having the power of a villain. However, many of the effects are overestimated and widespread even in people who have no sensitivity to this constituent. For this reason, the use or withdrawal of gluten in the diet has generated a lot of controversy.

Note that the problem in consuming foods that have gluten is not in protein intake, but in excessive consumption and the lack of other sources of nutrients. Before criminalizing a food or a specific constituent and cutting it from the diet for health maintenance, maintaining a balanced diet is essential. Many nutritional problems and obesity are due to an unbalanced diet and lack of physical activity.

Gluten itself in healthy individuals does not hurt; the problem is that baked goods are accompanied by other ingredients rich in sugars and fats (Kulai and Rashid, 2014). Thus the gluten-free diet can be considered beneficial for apparently healthy individuals and can also affect the health of the digestive tract. Individuals showed an increase in fat and calories from ingestion of a gluten-free diet (Zuccotti et al., 2013; Miranda, 2014), as well as obesity, overweight, recent insulin early resistance, and metabolic syndrome (Kabbani et al., 2012; Tortora et al., 2015). A gluten-free diet can also lead to vitamin deficiencies of B complex, folic acid, and iron (Shepherd and Gibson, 2013). However, the gluten proteins are well known to cause allergic responses in some individuals with celiac disease and, therefore, many people develop some type of sensitivity to this constituent.

Thus, there is no data to support the theory of an intrinsically toxic property of gluten. Gluten, gliadins and glutenins, is one of the most diverse wheat protein components, and for most people these proteins do not lead to any disease or reaction.

With respect to gastrointestinal health, there is speculation on the prebiotic action of gluten. It is possible to see differences in the intestinal tract of individuals with gluten restriction when compared to the microbiota of a healthy person (Jackson, 2010). The exclusion of foods containing wheat reduces the proportion of beneficial bacteria that colonize the intestine, possibly because of reduced intake of fiber in cereals. Because of the prebiotic effect of the consumption of cereals, especially whole cereals, contributes to improving health rather than harm it.

The sensitivity developed by some individuals when eating certain foods can be caused by food intolerance or allergy. Food intolerance is defined as any sensitivity to the food, including toxic, pharmacological, metabolic or

idiosyncratic reactions, since they are not caused by immunological mechanisms. Food allergy is a noninfectious reaction, an abnormal immune response to a food or constituent, which causes damage to the body (Mahan and Scott-Stump, 1998). For celiac, the immunological response takes place in the presence of prolaminna fraction. There is common confusion between the terms used; for Mahan and Scott-Stump (1998), the symptoms of allergy and food intolerance are often the same. Therefore, celiac disease is considered a food allergy; it involves immunological mechanisms, although many authors use the term "food intolerance" (Preichardt and Gularte, 2013).

4. CELIAC DISEASE

Celiac disease is a condition of the gastrointestinal tract which results in the inability to properly digest the gluten prolamin, called gliadin in wheat. It is a gluten allergy, considered an autoimmune disease or gluten-sensitive enteropathy, tropical sprue or nonsprue (Murray, 2002; Mahan and Scott-Stump, 1998).

This disease is the product of interaction of environmental, genetic and immunological factors causing an inflammatory lesion in the gut, affecting the middle and proximal portions of the small intestine, causing intestinal microvilli atrophy and malabsorption of nutrients (Baptista et al., 2005). The immune reaction to gluten appears commonly in infancy and causes irritation and damage to the lining of the small intestine, making it totally unable to absorb specific nutrients, which is a particular concern because of the damage during the growth phase in this age group. Symptoms of celiac disease can also appear later in life and are associated with maldigestion and malabsorption of nutrients, minerals, and vitamins in the gastrointestinal tract due to a toxic gluten effect that damages the gut villi causing diarrhea, fatigue, and weight loss (Mahan and Scott-Stump, 1998; Vilppula et al., 2011).

In celiac patients the toxic fraction of the gluten is prolamin, found in wheat, barley, rye, and oats. The cause of toxicity is the high content of glutamine amino acid (>30%) and proline (>15%) in these cereals. This behavior is not observed with the prolamins of rice and maize, in which the predominant amino acids are alanine and leucine. There is no consensus on oat toxicity; this cereal has a small amount of avenin (4%-14% of total protein) compared to approximately 40% wheat gliadin. Moreover, the composition of avenin has a middle quantity of glutamine and proline amino acids and

therefore only an excessive intake of cereal could cause damage (Dani, 2001; Mäki and Collin, 1997).

A deficiency of a specific peptidase mucosa in patients with celiac disease causes the villi of the intestinal mucosa to become smoothed and flattened in the presence of gliadin. It is believed that a receptor on the surface of the intestinal cells allows the gliadin to join the enterocyte and this complex gliadin/receiver, promoting inflammation, which causes atrophy of the villi and hyperplasia of the intestinal crypts, damages the mucosa, and causes antigenic reactions, triggering production of antibodies and the appearance of symptoms characteristic of the disease (Murray, 2002; Mahan and Scott-Stump, 1998). To Utiyama, Reason, and Katze (2004), patients with celiac disease have an increased intestinal permeability, and gliadin peptides are not fully degraded by intraluminal peptidases and can penetrate the epithelium and drive immune responses. In celiac patients intestinal mucosal changes are observed after stimulation with gliadin, because the involvement of T cells that are the main effectors of cellular immunity and recognize the antigen-specific and produce cytokines in the epithelium (Guandallini Gupta 2002).

The genetic predisposition to celiac disease may be for the most part related to the human leukocyte antigen (HLA) DQ2 and DQ8 haplotypes. Approximately 40% of the genetic risk of developing celiac disease is the responsibility of HLA-DQ genes. Genome studies have also reported an association with susceptibility to development of celiac disease by 39 additional non-HLA regions (Dubois et al., 2010). Not all of these gene carriers who develop the disease, however, approximately 98% of celiac patients present histocompatibility antigen HLA heterodimers determined by HLA-DQ2 or HLA-DQ8 (Utiyama, Reason, Katze, 2004). According to Nisticò et al. (2006), co-occurrence of the disease in monozygotic twins is only 85%. Therefore other genetic, immunological, and environmental factors must be involved.

Gluten allergy was considered for many years a pediatric disease. However the diagnosis in adults has been increasingly frequent, and it is now considered that it can occur at any age. Recent advances have been made in the knowledge of intestinal inflammatory response in celiac disease, and due to improvements in diagnostic tools, an increased prevalence of celiac disease worldwide has been observed (Kang et al., 2013).

Celiac disease mainly affects individuals of Caucasian origin and reaches most often the Anglo-Saxon and Nordic countries. Prevalence in the UK and continental Europe is 1/100, while in North America the ratio is 1/300. Several authors suggest that the disease affects twice as many women than men; others

maintain an equal distribution between the sexes. Among identical twins agreement is 70%-100%. The probable prevalence among first-degree relatives is 10%-15%. It is a disease with common prevalence in individuals with other autoimmune diseases such as type 1 diabetes mellitus and thyroid diseases (Ciclitira, 2003). In Brazil, due to high marriage between people of different backgrounds, this disease has been described even in African-Brazilians. It is evident, however, to be more prevalent in the South and Southeast, where in addition to having the largest population density of Caucasian origin, there is also a greater range of diagnostic tools (Kotze, 2005).

The most common symptoms of celiac disease are bloating, irregular bouts of diarrhea, abdominal pain, and dermatitis herpetiformis; however, symptoms may vary depending on the stage of the disease. Some symptoms mimic other diseases, such as parasitic infections, anemia, gastric ulcers, and IBS. Unspecific symptoms may also occur, such as skin rash, dry mouth, dental problems, bone problems, depression, irritability, stomach cramps, and neuropathy (tingling in the arms and legs) (Ciclitira, 2003; Mahan and Scott-Stump, 1998).

Several studies to decrease or eliminate the symptoms of gluten allergy are developed by producing monoclonal antibodies, DNA technology, cell cloning, and food engineering. Based on this, experiments have been developed with application of oral supplements of special enzymes, compounds that block the adhesion site on the HLA molecule, and other vaccines (Preichardt and Gularte, 2013).

If left untreated, celiac disease can lead to gastrointestinal adenocarcinoma and lymphoma of the small intestine. What can prevent complications caused by the disease is a strict diet free of gluten (Benahmed et al., 2003; Coultate, 2004; Gallagher, Gormley, Arendt, 2003). Accordingly, the food industry, especially in food producing gluten, must have strict control of processing to avoid cross-contamination with products that contain wheat, both in the choice of raw material and the cleaning of equipment. It is important to the establishment and enforcement of laws to ensure the health of celiac patients, as well as the promotion of standardization and validation of methods for gluten detection by scientists (Possik et al., 2005).

Celiac patients can transgress dietary requirements, so we need to know the disease and its treatment. The information described correctly on food labels is important to prevent the unintended use of this constituent in industrialized products. This type of accident can happen, from harvesting of

the raw material until the time of sale of the food (Preichardt and Gularte, 2013).

5. OTHER GLUTEN-RELATED DISORDERS

In celiac disease, other eating disorders related to gluten have been reported by researchers. Among them: dermatitis herpetiformis, gluten ataxia, wheat allergy, and nonceliac gluten sensitivity (Foschia et al., 2016). Table 1 summarizes the prevalence and symptoms of gluten-related disorders. The table, prepared by Foschia et al. (2016), is based on a compilation of studies and works by the author.

Table 1. Prevalence and symptoms of gluten-related disorders

Disease	Prevalence in the world (approximate values)	Symptoms
Celiac disease	1.0%	Chronic diarrhea, failure to thrive, fatigue, malabsorption, anemia
Nonceliac gluten sensitivity	3.0%-6.0%	Gastrointestinal complaints, weight loss, bloating, diarrhea, muscular disturbances, bone pain, tiredness, neurological disorders
Wheat allergy	0.5%-9.0%	Urticaria, angioedema, erythema, dyspnea, oropharyngeal symptoms, urticaria, angioedema, atopic dermatitis flare, rhinitis, asthma, gastrointestinal symptoms, anaphylaxis, pruritus, eczema
Dermatitis-herpetiformis	0.0001%-0.0500%	Urticarial plaques, blisters on the elbows, buttocks, and knees
Gluten ataxia	14.0%	Insidious onset of predominantly gait ataxia, often associated with symptoms and signs suggestive of peripheral neuropathy
Crohn's disease[a]	0.0007%-0.0199%	Fever, weight loss, diarrhea, abdominal pain
Irritable bowel syndrome[a]	5.0%-30.0%	Similar to CD and NCGS, bloating, diarrhea, gas, and abdominal pain

[a]Gluten-free diet often recommended, but gluten is not the cause of the disease.
Source: Foschia et al. (2016).

Dermatitis herpetiformis is also known as Duhring-Brocy disease and often called celiac disease of the skin (Kárpáti, 2015). This disease is characterized by urticarial plaques and blisters on elbows and buttocks. Other parts of the body can also be affected (Caproni et al., 2012).

Gluten ataxia is also an immune disease triggered by the ingestion of gluten in genetically susceptible individuals (Hadjivassiliou et al., 2015). It can be further defined as idiopathic sporadic ataxia in the presence of circulating anti-gliadin antibodies (IgA/IgG) (Hadjivassiliou et al., 2008). The immune response is mediated by IgE and affects the gastrointestinal tract, respiratory system, or skin (Keet et al., 2009; Tatham and Shewry, 2008).

The term "sensitivity to gluten not celiac" (NCGS) has been used to name the symptoms that many people develop, both gastrointestinal and nongastrointestinal, which are ameliorated when gluten is excluded from the diet (Catassi, 2013). Another term used is "not celiac sensitivity to wheat," assuming that gluten is not responsible for the symptoms (Carroccio, Mansueto, 2013; Mooney, Aziz, Sanders, 2013).

Different from what occurs in celiac disease, NCGS occurs in the gastrointestinal tract with permeability of the intestinal barrier preserved; histological changes of the villi and crypts are not severe, with, however, introduction of numerous disorders in a patient maintaining a conventional diet rich in gluten. Still, there is mild infiltration of lymphocytes and a lesser degree of inflammation in the intestinal tract compared to that observed in individuals with celiac disease. However, there are no data to show that withdrawal of gluten eliminates the symptoms, since exclusion of foods high in nonabsorbable sugars and polyoils (FDMAPS) can improve symptoms, regardless of gluten ingestion (Biesiekierski et al., 2013), making the NCGS condition similar to that of IBS (Verdu, Armstrong, Murray, 2009). IBS is a common functional gastrointestinal disorder characterized by recurrent abdominal pain or discomfort associated with altered bowel habits. Despite being a benign disease by definition, IBS takes a chronic relapsing-remitting course with associated fatigue, depression, anxiety, and decreased quality of life (Chey, Kurlander, Eswaran, 2015).

Table 2 describes characteristics of the main gluten-related disorders (information compiled by Drabińska, Zieliński, Krupa-Kozak, 2016).

Table 2. Characteristics of gluten-related disorders

Characteristic	CD	WA	NCGS
Definition	Immune-mediated enteropathy triggered by ingestion of gluten in genetically susceptible individuals	Adverse immunological reaction to wheat proteins	Gluten sensitivity with negative immunoallergy tests to wheat or negative CD serology with normal duodenal histopathology
Morbidity	≈1%	≈1%	Unknown
Genetic background	HLA-DQ2/DQ8	Atopy	HLA-DQ2/DQ8
Triggering proteins	Gluten	Gluten/nongluten	Gluten/nongluten
Symptoms	Intestinal and extraintestinal	Intestinal and extraintestinal	Intestinal and extraintestinal
Diagnosis	CD symptoms, positive CD serology, determination of HLA-DQ2/DQ8, small-intestine biopsy	Specific skin prick tests, wheat-specific serum IgE assays, gluten challenge	Exclusion criteria for CD and WA, gluten challenge
Serology	IgA/IgGe	IgE	IgG
Atrophy of duodenal villi	Present	May be present	Absent
Treatment	Gluten-free diet	Wheat-free diet	Wheat-/gluten-free or reduced diet
Time of diet duration	Lifelong	Average 6 years. Lifelong in anaphylaxis	Unknown

CD – celiac disease; WA – wheat allergy; NCGS – nonceliac gluten sensitivity;HLA – human leukocyte antigen; Ig – immunoglobulin.

Source: Drabińska, Zieliński, Krupa-Kozak (2016).

6. TREATMENTS

In general, a strict gluten-free diet has been recommended for all disorders related to gluten, such as celiac disease, dermatitis herpetiformis, celiac nonsensitivity to gluten, and gluten ataxia (Pietzak, 2012). For celiac disease there is no cure, but the disease can be controlled and intestinal damage prevented through a proper diet.The most effective treatment to prevent complications caused by celiac disease is to not consume foods containing gluten. It is extremely important to maintain compliance with the gluten-free

diet in order to ensure height and weight and pubertal development suitable, bone mineral density, fertility, well as reduces risk of digestive system disorders (Sdepanian et al., 2001). Therefore, treatment is basically dietetic,the patient must remove gluten from the diet throughout life. The diet of people with celiac disease should meet age-appropriate nutritional needs.

After removal of dietary gluten, symptomatic clinical response is rapid, resulting in disappearance of gastrointestinal symptoms within days or weeks. Histological changes of the intestinal mucosa take longer. Reduction of intraepithelial lymphocytic infiltration occurs within a few weeks, but recovery of normal villous appearance may take two to three months. Despite the improvement of symptoms and well-being, some patients never recover fully (Sdepanian, Morais, Fagundes Neto, 1999). The return of normal or nearly normal mucosa with strictly gluten-free diets is possible; however, changes of the intestinal mucosa are repeated after their reintroduction in the diet (Dani, 2001), even after many years of avoidance (Koehler et al., 2014). The goal of treatment is to improve the quality of life of patients, reverse the changes of the enteric mucosa, adjust nutritional deficiencies, and reduce the risk of developing lymphoma of the small intestine.

In some cases, the natural balance of microbiota in patients suffering from gluten sensitivity may not be completely restored (Nadal et al., 2007). Furthermore, the use of gluten-free diets can modify the composition and immunological properties of intestinal microbiota in adults (Marasco et al., 2016). It is possible that gluten contributes a prebiotic action in the intestine; a gluten-free diet can induce intestinal microbiota of a different composition compared with a healthy person (Jackson, 2010).

As the only treatment for celiac disease is a gluten-free diet, celiac patients must maintain a strong commitment to diet and self-educate regarding food and food ingredients. The diet's staple foods are fruits and vegetables, cereals, grains, tubers, dairy products, and meat, provided that monitor the labels to make sure that it is gluten-free foods.The foods that normally have gluten are baked goods (breads, cakes, cookies, pasta), sauces, and sausages (Preichardt and Gularte, 2013). The market has available several gluten-free products that celiac patients can consume. However, patients have to follow rules and be aware of sources of "hidden" gluten, because this constituent is also used as a functional ingredient in sauces, meat, and fish products (Day et al., 2006; Kalin, 1979).

Another issue to be observed is that many products do not naturally contain gluten but may be contaminated and show traces of proteins due to the production process. In order to avoid any kind of contamination, the

ingredients containing gluten in the composition should be stored and handled in separate areas from products free of gluten. On this basis, it is recommended that patients suffering from diseases related to gluten must observe the labeling and certification of products, as well as check analytically and regularly possible gluten residues in certain products (Thompson and Simpson, 2015).

FINAL CONSIDERATIONS

The growth of the gluten-free products market was not due to the increased incidence of diseases related to gluten or greater rigor in the treatment of patients, but rather to increased demand generated by adherence to a diet that is gluten-free by individuals that are nonceliac and not diagnosed with NCGS. This is the main reason people eliminate gluten from the diet, the belief that gluten-free products would be healthier than with use of traditional products with gluten, would aid in weight loss, and would gain improvement in other pathophysiological conditions and gastrointestinal discomforts.

Despite significant improvement in the health of individuals with changes related to eliminating consumption of gluten, it appears that there is insufficient evidence to support the belief among individuals who are not sensitive to this protein fraction that a gluten-free diet is beneficial. Celiac patients may gain weight due to the gluten-free diet, often because of poor dietary intake in whole grains and fiber and increased intake of fats and sugars. Additionally, the prebiotic action of gluten has been studied showing that the consumption of cereals, especially whole grain cereals, contributes to improvement of health rather than harming it.

Therefore, the gluten-free diet should be recommended for people who have an allergy or sensitivity. In these cases, the exclusion of dietary gluten is the only form of treatment; and through the restrictive diet it is possible to control intestinal damage and improve quality of life of patients.

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Chapter 5

TECHNOLOGICAL FUNCTIONS OF GLUTEN AND IMPLICATIONS FOR CELIAC DISEASE

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ABSTRACT

Gluten and gluten-related proteins (prolamin and glutelin) may be present in several cereals, such as wheat, rye, barley, oat and the derivatives of these grains, including malt and brewer's yeast. Consequently, several commercial foods contain gluten proteins in its composition, for example: pastas, cookies, breads, gravies, salad dressings, soups, processed lunchmeats and snack foods. Despite of some specific health implications, cereals are important carbohydrate and proteins source for human diet. Phenolic acids, vitamins, minerals and dietary fiber also can be found in wholegrains. Nowadays, cereals have been investigated about its potential use as ingredient in functional foods. Therefore, the development of food products with health benefits is a challenge for the food industry. The consumers demand for food with

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sensorial quality and suitable to specific restrictions have encouraged the diversification of general cereal-based foods intake.

Gluten is the main structure-forming protein, primarily in wheat flour, composed for two different proteins: gliadin (a prolamin fraction) and glutenin (a glutelin fraction). An acceptable definition for the protein complex is "cohesive, viscoelastic proteinaceous material" that remains when wheat dough is washed to remove starch granules and water soluble constituents. Focused on wheat flour proteins, monomeric gliadins and polymeric glutenins (*high molecular weight glutenin subunit* - HMW-GS and *low molecular weight glutenin subunit* - LMW-GS) impart distinct functions on gluten formation, depending on capable to form intra- and inter-chain disulfide bonds. The properties of gluten become apparent when flour is hydrated, giving elastic and viscous characteristics to dough, with good gas holding properties, and a good crumb structure in baked bread. Gluten is often termed the 'structural' protein for breadmaking being largely responsible for end-use quality of wheat in many food products. The absence of gluten results in major problems for bakers, and currently, many gluten-free products available on the market are of low quality, exhibiting poor mouthfeel, texture and flavor.

Celiac disease (CD) is the main health gluten implication and the most common food induced enteropathy in humans. CD is defined as an inherited autoimmune disorder that affects the digestive process of the small intestine that develops in predisposed subjects, and is triggered by ingestion of gluten. Celiac disease is a life-long intolerance to the gliadin fraction of wheat and the prolamins of rye (secalins), barley (hordeins) and possibly oats (avidins). Celiac disease affects 1 in 100-200 individuals, this prevalence is significantly higher than that recognized years ago. The effective treatment for celiac disease is a gluten-free diet throughout one's lifetime, which results in clinical and mucosal recovery, when followed closely. However, patients can present other implications that are not celiac disease as allergy to gluten which activates the IgE (immunoglobulin E) and consequently histamine, mast cells and basophils, causing the allergic attack. A change in intestinal permeability gives rise to the passage of antigens which leads the onset of diseases including those related to gluten.

INTRODUCTION

Gluten is a complex mixture of proteins comprising the gliadins and glutenins, which are present in cereals such as wheat, rye, triticale, barley and oat. However, wheat gluten is mostly studied because of unique properties of network formed in water presence that gives special viscoelastic

characteristics to dough. Therefore, the majority of the processed food products of human diet comprise one or more cereals gluten source in their formulations. Baked goods such as bread, cake, biscuits and crackers, besides pasta, require wheat flour/semolina to achieve the specific technological characteristics.

For many years the studies have showed and got the bottom of the importance of High Molecular Weight glutenin subunit (HMW-GS) on rheological dough properties and bread quality. Continuously, distinct ways to improve the properties of the cultivated around the world have been explored. Generally, environment, genetic and fertilization are factors influencing the gluten properties.

Despite of technological importance of gluten on structure of leaven bakery products, as bread, gluten proteins also have negative impacts on human health, in relation to allergies and intolerances. Three pathologies are associated with gluten intake, which appear to be increasing in importance: a) food allergy, b) coeliac disease (CD) and c) gluten sensitivity. The methods for gluten determination are continuously investigated to guarantee the enhanced sensitivity of the detection techniques, considering the protein complexity structure, mainly in processed foods. The Codex Commission Review 2004 recommended the limits of 20 mg/kg dry mass for naturally gluten- free products, and 200 mg/kg for food derived from gluten free products.

CD has a worldwide distribution, being described in different ethnic groups from North and South America, Europe, south and west Asia, Australia and New Zealand (Fasano and Catassi, 2012). CD is a chronic small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals (Ludvigsson et al., 2013). Currently studies have been explored immunopathogenetic targets as alternative treatments: intraluminal digestion of gluten, improvement in barrier functions, and immunomodulators (BAI et al., 2008). However, the only effective treatment for all CD patients is a gluten-free diet (Demelo et al., 2015).

The last decade was crucial by bringing to the attention of gluten-free food. Ever since, different ingredients has been used with the purpose to overcome the sensory and textural difficulties associated to gluten-free food manufacturing, such as: hydrocolloids, gum, ancient flours and fibers.

This chapter was organized in three sections. In the first section we will approach some general information about gluten food sources, natural and processed one, and the advances in gluten detection. In the second section we will focus on gluten properties. Lastly, we will provide some discussion about celiac disease: pathology, diagnosis and treatment.

1. FOOD SOURCES

The cereals represent the major component of the human diet in the world. They are important source of energy and protein, contribute to minerals and vitamins intake, and are rich in essential fatty acids and fiber. The most of the cereals are also gluten source, such as: wheat, rye, barley, oat and the derivatives of these grains, including malt and brewer's yeast (Green and Cellier, 2007; Gregor and Sey, 2010).

The gluten is a mixture of proteins classified into two groups, the prolamins and the glutelins. The prolamins and glutelins comprise 80-90% of the wheat, rye and barley proteins, respectively. The prolamins have specific names in the group of the cereals: gliadins in wheat, hordeins in barley, secalins in rye, zeins in maize and avenins in oat (Tatham and Shewry, 2008).

1.1. Cereals

1.1.1. Wheat

The wheat (*Triticum aestivum* L.) is widely consumed by humans as a source of calories. The grain is characterized for a high starch content which comprises about 60-70% of the whole grain and 65-75% of white flour. Wheat is an important source of vitamins and minerals of the human diet (Adhikariet al., 2016). The protein content is approximately 8-15%. In addition, wheat protein develops functional properties, mostly determined by the wheat gluten proteins which play a significant role in breadmaking. The gluten-forming proteins consist in two types, gliadins and glutenins, which are important to improve the functionality and quality of the food products (bread, some biscuits, pasta, among others) (Johansson et al., 2013). Generally, glutenin is responsible for gluten elasticity and the gliadin contributes with viscosity (Delcour et al., 2012). The mentioned "gliadins" (prolamin fraction) and glutenin (glutelin fraction) are found in wheat kernel in the range of 33-45% and 40-46%, respectively (Eliasson et al., 1993).

1.1.2. Rye

The rye (*Secale cereale* L.) composition is similar to wheat. Generally, rye contains less starch and crude protein. On the other hand, the cereal contains more dietary fiber which includes: arabinoxylan, β-glucan, cellulose, fructan and lignin. Rye is also a source of a wide range of micronutrients and

bioactive compounds. The use of whole meal rye flour in bakery products formulations has increased with the advantage to provide considerable levels of dietary fibers, vitamins, minerals and phytoestrogens (Verwimp et al., 2007; Poutanen et al., 2014). The prolamin fraction in rye is called secalin, whilst glutelin fraction is denominated secalinin. They are found in rye grain in the range of 25-40% and 33-42%, respectively (Eliasson et al., 1993).

1.1.3. Barley

Barley (*Hordeum vulgare* L.) is the fourth largest cereal crop in the world both in terms of quantity produced and area of cultivation (Xia et al., 2012). Nutritional components of barley include starch, fiber, proteins, lipids, vitamins, minerals, and phytochemicals (Newman and Newman, 2008). Protein is the second most abundant component (8–13%) in barley grain after starch (60-65%) (Wang et al., 2010). The hordein, prolamin fraction of the barley, represents 25-52% of the grain (Eliasson et al., 1993). Glutelin, a major fraction of barley protein (35–45% of the total storage protein) (Lâsztity, 1995), is characterized by high proportions of glutamine (glutamic acid, 20.2%), proline (11.2%) and glycine (8.4%) (Wang et al., 2010).

Malted barley is recommended for beer brewing which is produced from the whole barley grain. It is increasing the interest in malt from sorghum and pseudo-cereals for use in gluten-free beers (Schwarz and Li, 2011).

1.1.4. Oat

According to Codex standard, oat (*Avena sativa* L.) is a gluten-containing cereal as wheat, rye and barley. The majority compounds found in oat are dietary fiber, protein, unsatured fatty acids and bioactive compounds (Sontag-Strohm et al., 2008). In oats, the globulins are predominant and comprise 50–80% of the seed protein. The prolamin fraction is called avenin and represents around 10-20%, while the glutenin fraction content is 12-14% of the seed protein (Eliasson et al., 1993; Barbosa et al., 2007). However, the the avenins share the basic feature of other cereal prolamins as being polypeptides relatively rich in proline and glutamine. These regions of the amino acid sequences are associated with the elicitation of celiac disease (Armstrong et al., 2012). Although, oats are considered generally a safer alternative to other cereals where the prolamins are the dominant seed protein (wheat, barley, rye, maize, sorghum), it is not clear which are the existence and prevalence of celiac eliciting epitopes in the oat avenins (Real et al., 2012).

All these cereals are used in the food industry as ingredient to produce different processed foods, as described in Table 1.

Additionally, other important gluten sources includes: (a) any bread, cereal or other food made with wheat, rye, barley, triticale, dinkel, kamut and oat flour or ingredients, and by-products made from those grains; (b) processed foods that contain wheat and gluten-derivatives as thickeners and fillers, for example hot dogs, salad dressings, canned soups/dried soup mixes, processed cheese, cream sauces; and (c) medications that use gluten as pill or tablet binders (Gallagher et al., 2004).

Consumers are increasingly concerned about the food composition and they are interested to intake food products naturally high in macronutrients and micronutrients. Those requirements fully apply to cereal-based goods (Guine and Correia, 2013). Due the linkage of gluten with some health implications the knowledge of gluten detection in foods s is the industry interests and consumer health safety.

Table 1. Cereal based food products

Cereal	Product
Wheat	Specially processed food products from flour and other parts of the wheat kernel as germ, bran and endosperm.
Rye	Food products made from all parts of rye grain, mainly flour. Breadmaking is the primary use.
Barley	Food products made from barley flour and its parts as malt flavorings, brewer's yeast and beer.
Oat	Food products containing oat flour, oat bran and oat gums such as bread, pasta, snacks, drinks, breakfast cereals and biscuits.

Source: Buhs-Catterall (2010), with modifications.

1.2. Gluten Detection in Foods

In recent decades, gluten has attracted great attention due to the increasing number of diagnosed patients with intolerance to this protein fraction, relating to the improved sensitivity of the detection methods and the increasing awareness of the existence of the disease. Since the only effective treatment is the avoidance of gluten containing foods, it is necessary to have reliable methods for gluten determination (Rosell et al., 2014).

The presence of the numerous gluten components, the variation in the extraction efficiency, and the lack of reference materials are some issues that hinder the implementation of equivalent laws at national level and the

comparison of data across the different methods (Diaz-Amigo and Popping, 2012). Hydrolysis and deamidation widely used processes in the food industry and protein aggregates formed by heat-treatment of cooked and baked products makes gluten analyses even more difficult (Rosell et al., 2014).

Important factor in prolamin and glutelin analysis is complete extraction from the samples. Therefore, it is necessary to use an extraction system giving complete recovery of both prolamins and glutelins, to guarantee that products marketed as suitable for celiacs are really gluten-free. The extraction of gluten from wheat, barley, rye and oat standards is easier than from processed foods (Rosell et al., 2014). More recently, combination of agents called UPEX (universal prolamin and glutelin extractant solution) leads to a complete extraction and it is compatible with all gluten analysis procedures (Mena et al., 2012). In addition, a novel universal gluten extraction solution (UGES) has recently been described (Biomedal Diagnostics, Seville, Spain). The UGES procedure gave high extraction efficiency from both simple and complex matrices even if they had heat-processed (Rosell et al., 2014).

Another critical point in gluten analysis is the use of a correct standard. This standard should be as representative as possible of the gluten proteins to be analyzed (Rosell et al., 2014).

The most practical methods for food analysis are based on immunological analysis. Some methods including the polymerase chain reaction (PCR), HPLC and MS, may be used simultaneously with immunological methods. One of the most recent methods developed for gluten analysis is a potentiometric electronic tongue, which was able to detect 1e 2 mg/kg of gliadin from different matrices (Peres et al., 2011).

2. GLUTEN PROPERTIES

Gluten is the main structure-forming protein (network) in flour, and is responsible for the elastic characteristics of dough, and contributes to the appearance and crumb structure of many baked products. The properties of gluten become apparent when flour is hydrated, giving an extensible dough, with good gas holding properties, and a good crumb structure in baked bread (Gallagher et al., 2004). Wheat flour is almost unique amongst cereal flours with properties that enable it to be used for preparing an extensive range of leavened baked products that the consumer finds attractive. These properties are due to viscoelastic properties of storage proteins (gliadin and glutenin). This is found to a lesser extent in rye, even less so in barley and is entirely

absent from maize and sorghum (Attenburrow et al., 1990). Based on that, in this session we will give attention to wheat gluten.

Wheat gluten contains the protein fractions glutenin (wheat glutelin) and gliadin (wheat prolamin). When fully hydrated, gluten exhibits cohesive, elastic and viscous properties that combine the extremes of the two components. Quantity and quality of gluten protein are important parameters for end-use quality of wheat (Zhang et al., 2007). When dough is developed by mixing or sheeting, the gluten protein forms a continuous viscoelastic network throughout the dough (Singh and MacRitchie, 2001). These properties make wheat alone suitable for the preparation of a great diversity of food products: breads, noodles, pasta, cookies, cakes, pastries and many other foods.

2.1. Gluten Protein Composition and Quality Properties

Gluten comprises approximately equal amounts of gliadin and glutenin proteins, representing 80% of total grain proteins, and contains high levels of amino acid glutamine (DAY et al., 2006). The gliadins are monomeric proteins which interact by non-covalent forces (notably hydrogen bonds) (Shewry et al., 2003). The glutenin polymers comprise two groups of subunits linked by inter-chain disulfide bonds. These are called low-molecular-weight glutenin subunits (LMW-GS) and high-molecular-weight glutenin subunits (HMW-GS). A further definition might include the genes involved in the synthesis of the gluten proteins in the developing grain—the Gli-1 and Gli-2 loci coding for the gliadin proteins, and the Glu-1 and Glu-3 loci, coding for the glutenin polypeptides (Gianibelli et al., 2001).

The glutenin subunits explain the variation of dough and gluten properties better than the content of gliadins (Wieser and Kieffer, 2001). Specially, HMW-GS plays an important role in determining the gluten network structure (Don et al. 2006). Single loci encoding HMM subunits are present on the long arms of the group 1 chromosomes (1A, 1B, 1D), each locus comprising two genes encoding subunits that differ in their properties and are called x-type and y-type subunits (PAYNE, 1987). The contributions of x-type HMW-GS to dough rheological properties are much higher than those of y-type HMW-GS (Wieser and Kieffer, 2001). Analyses of such lines have confirmed that the subunits are largely responsible for determining dough viscoelasticity and that specific allelic subunit pairs are associated with either high or low dough strength.

Great breadmaking quality is particularly associated with the presence of a 1Ax subunit (compared with the silent or null allele) and the chromosome 1D-encoded subunit pair 1Dx5+1Dy10 (compared with the allelic subunit pairs 1Dx2+1Dy12, 1Dx3+1Dy12 and 1Dx4+1Dy12) (Shewry and Halford, 2002). The presence of HMW-GS "5" is accompanied by a good breadmaking quality, whereas HMW-GS "2" by a poor quality (Shewry and Tatham, 1997; Payne et al., 1987). The progressive studies conclude that a different polymerization behavior via intermolecular disulphide bonds could be responsible for these quality differences. Despite of specific HMW-GS, amount and proportions of gluten protein types essentially determine gluten quality.

2.2. Gluten Properties in Dough Formation

The importance of gluten in breadmaking technology concerns to cohesive-elastic dough obtained by the flour and other ingredients incorporated to concentrated systems (Sgarbieri, 1996). Three factors are required to form dough: flour, water and energy. Shear and tensile forces imparted by mixing or sheeting cause discrete masses of gluten protein to coalesce and form a continuous network throughout the dough. During development, the dough acquires viscoelastic properties which become optimum at peak consistency.

Gluten proteins are largely responsible for important rheological properties of dough (extensibility, resistance to stretch and mixing tolerance), and consequently, for the baking quality of wheat. Gluten is important to retain gas to obtain the desired volume and texture in a dough system. The rheological properties of gluten enable breads, cakes, biscuits, and noodles to be made from wheat flour.

Gliadin and glutenin fractions are important contributors to the rheological properties of dough, though their functions are divergent. While prolamin provides viscosity and extensibility in a dough system, glutenin is responsible for elastic and cohesive properties of dough. HMW-GS and LMW-GS proteins interact to make dough elastic, which allows dough to trap the gas bubbles produced by yeast, which in turn causes bread to rise (Shewry and Halford, 2002).

The viscoelastic properties of gluten are related to the ratio of glutenin to gliadin and to the proportion of larger polymeric proteins, which have also been classified as glutenin macropolymer (GMP) or sodium dodecyl sulfate

(SDS)-unextractable polymeric proteins (UPP). Studies have shown that the unextractable polymeric proteins (UPP), is the main contributing to dough strength (KOGA et al., 2016).

2.3. Environment Factors Influencing Gluten Properties

Both genetic and environmental factors such as temperature, nutrient availability (particularly of sulfur and nitrogen) and water availability are reported to influence the properties of gluten proteins (Altenbach, 2012). The effects of temperature on the viscoelastic properties of gluten were reported to be associated with changes in the assembly of large glutenin polymers rather than changes in the composition of gluten proteins.

The environmental conditions and the use of different agronomic practices can get to influence more on wheat grain N concentration than the genetic or varietal differences (Fuertes-Mendizábal et al., 2010). In relation to agronomic practices, nitrogen nutrition is largely considered as the main factor affecting storage proteins as well as the technological quality of the grain. As demonstrated in some studies both the different kinds of N fertilizer and the application techniques affect the amount of storage proteins (Garrido-Lestache et al., 2005).

The ratio of high molecular weight glutenin subunits (HMW-GS) to low molecular weight glutenin subunits (LMW-GS) increased in response to fertilizer, due in part to small increases in the proportions of individual HMW-GS **(**Altenbach et al., 2016)**.** Also, high HMW-GS and LMW-GS were improved by sulphur fertilizer under lower nitrogen (N).

2.4. Gluten Replacement in Bakery Products

As the theme explored, gluten is an essential structure building-protein, being necessary for formulating high quality cereal-based goods. This characteristic is usually provided by unique wheat flour gluten. Considering the increase in gluten free products demands and apparent or real celiac disease, or other allergic reaction/intolerances to gluten, the researchers, technologists and bakers are being stimulated to find alternatives to gluten in the manufacturing gluten free bakery products of high quality. Therefore, to produce baked goods with gluten replacement is still a technological challenge.

The term ‘gluten-free foods’ refers to food products free from storage proteins of wheat, rye, triticale, barley and possibly oats, or those in which cereal protein content is less than 200 ppm (Kasarda, 2001).

The absence of gluten often results in a liquid batter rather than dough pre-baking, and can result in baked bread with a crumbling texture, poor color and other quality defects post-baking (Matos and Rossel, 2013). Besides the addition of different components by different flour and dough treatments, changes in the method of baking are required to improve quality in gluten-free bread production.

A desirable cake is characterized by a spongy texture attributed to the large number of small air cells, which provide the typical porous structure and high volume (Martínez-Cervera et al., 2012). Gluten-free cake products commonly contain rice flour as a basic ingredient. However, the rice presents low gas holding capacity and it is unable of forming networks (gluten absence). Proteins, hydrocolloids, resistant starch and emulsifiers can be added to the batter to improve final product quality (Turabi et al., 2008, 2010; Tsatsaragkou, 2014; Matos et al., 2014).

Gluten-free biscuits manufacturing did not represent hard difficult, as the development of a gluten network in biscuit and cookie dough is minimal and undesirable (apart from some semi-sweet biscuits, which may have a developed gluten system); the texture of baked biscuits is primarily attributable to starch gelatinization and super cooled sugar rather than a protein/starch structure (Gallagher, 2002).

Pseudo-cereals and non-gluten cereal grains like corn and rice are the staple for gluten free products. Gluten removal results in major problems for bakers, and currently, many gluten-free products available on the market are of low quality, exhibiting poor mouthfeel and flavor (Arendt et al., 2002). Diverse approach has been given to the use of starches, dairy products, gums and hydrocolloids, other non-gluten proteins, prebiotics and combinations, as alternatives to glute as way to improve the structure, mouthfeel, acceptability and shelf-life of gluten-free bakery products (Gallagher et al., 2004; Aguilar et al., 2015; Foschia et al., 2016). In association they mimic the viscoelastic properties and improve the nutritional profile of the food (Foschia et al., 2016).

3. HEALTH IMPLICATIONS

The main health implications caused for consumed of gluten are Celiac Disease (CD) and gluten-sensitive enteropathy. CD is common lifelong disorders with incidence in 1% of the population including many areas in the worldwide. It is recognized in every continent as Asia, Middle East, North Africa and South America. CD is associated to others autoimmune diseases such as diabetic, osteoporosis, arthritis, ulcerative colitis and Chron's disease, thyroid disorders and Addison disease.

Non-celiac gluten sensitivity is characterized for intestinal and extra intestinal symptoms related with the gluten containing food consumption which disappear with gluten withdrawal. It is an emerging novel entity as celiac diseases and irritable bowel syndrome. The small intestine is usually normal, but the individual presents symptoms similar to celiac disease (Fardet, 2015; Sapone et al., 2012).

3.1. Celiac Disease

Celiac disease is the end result of three processes: genetic predisposition, environmental factors and immunogically-based inflammation. CD is characterized by inflammation of the small intestine which adversely affects the absorption, leading to the malabsorption of several important nutrients including iron, folic acid, calcium and fat soluble vitamins (Sollid, 2000). CD is a result from the interaction between gluten intake and genetic factor HLA haplotypes DQ2 and DQ8 that take to immune response in the gut (Rubio-Tapia and Murray, 2010).

The only effective treatment for celiac disease is a strict adherence to a gluten-free diet throughout the patient's lifetime, which results in clinical and mucosal recovery (Dias, 2016). The habit of a gluten-free diet is essential to decreasing the symptoms (Morais and Alencar, 2015).

The gluten in celiac patients is resistant to a luminal proteolysis. When the gluten achieves the intestine and is recognized, inappropriate T-cell is activated and the intestinal mucosa is damaged by the action of inflammatory mediators and by matrix metalloproteinase enzymes secreted by stimulated fibroblasts (Woodward, 2015). A possible pathogenic mechanism involves increased permeability of the intestinal epithelium to gliadin (the immunogenic component of gluten) caused by dysregulation of the innate and adaptive immune systems (Rubio-Tapia and Murray, 2010). Intestinal cluster

of differentiation antigen 4 (CD4) T cells interact with gliadin peptides presented by human leukocyte antigens (HLA)-DQ2 or DQ8 and produce interferon-gamma, which leads to mucosal inflammation and structural and functional damage (Rostom et al., 2006). This cascade of inflammatory mediators promotes an inflammatory process characterized by crypts hyperplasia and villus atrophy. Individuals carrying HLA-DQ2 and HLADQ-8 as mayor genetic predisposition are affected (Brouns et al., 2013; Moscoso and Quera, 2015). The risk of celiac diseases could be caused by higher intake of food containing gluten at time weaning and introduction of food (Aronsson et al., 2015).

3.2. Clinical Presentation and Diagnosis

The clinical presentation of CD is varied between the patients. The symptoms include diarrhea, abdominal pain, anemia, osteoporosis, neurologic abnormality, increased liver enzyme levels, arthritis, iron deficiency and skin disorders (Green et al., 2015). Among the symptoms, some atypical presentations are encountered as neurologic problems. Females diagnosed with celiac disease have a history of infertility (Green, 2009). The treatment is a gluten-free diet which generally results in an improvement of the symptoms (Holtmeier and Caspary, 2006).

The rate of celiac disease diagnosis is increasing around the world. Specific serological test and histologic assessment of intestinal mucosa are used as a first step of diagnostic when there is suspicion of celiac disease. The capsule endoscopy is an alternative method used to identification of complications and mucosal damage. Genetic testing can also be used. The guidelines for celiac diseases have been proposed biopsy and serologic analyses for diagnosis (Kelly et al., 2015). In recent years the role of antibody testing has increased, because is a non-invasively that indicates the presence of gluten induced enteropathy. Gastroenterologists do not recommend biopsy diagnosis for adults with immunodeficiency conditions, extra intestinal manifestations and type-1 diabetes mellitus (Korponay-Szab et al., 2015).

3.3. Treatment and Gluten Free Diet

The gluten free diet is the only treatment for CD. The initiation of the treatment allows the mucosal recovery and minimizes disease symptoms

(Kelly, 2015; Volta et al., 2016). During the treatment is necessary follow-up of nutritionists and doctors with expertise in celiac disease is essential to ensure the treatment (Zarkadas et al., 2006). The gluten-free diet is a challenge mainly because gluten is widely found in most of common foods such as breads, cereals and pastas. Recent advances in food industry are making it easier by providing a larger variety of high quality gluten-free products (Niewinski, 2008). Pseudo-cereals are promising ingredients for gluten-free diets. Amaranth, buckwheat, quinoa and sorghum have been sold as whole seed milled in flour, flakes or grits (Lamacchia et al., 2014; Saturni et al., 2010).

The removal of gluten of celiac patient diet normalizes the intestinal morphology crypts and clinical symptoms are reduced. However, reintroduction gluten in the diet rapidly activates the pathogenic immune cells and subsequent infection of tissue destruction (Burger et al., 2016; Stamnaes and Sollid, 2015). The restrictive diet profile contrasts negatively with life quality and results in limitation in social life (Lee and Newman, 2003; Lovik et al., 2015). To obtain one balance of the gluten-free diet adoption are necessary tackling strategies in adolescent patients adherent and non-adherent to this diet. Psychological interventions may therefore be beneficial to support adolescents, between them, goal setting might be helpful (Wagner et al., 2016).

Recently, non-dietary treatments for celiac disease under investigation included intraluminal agents, immunomodulators, and vaccination. Between the purposes of these new treatments are to destroy toxic gluten peptides or to restore immune tolerance to gluten. Despite of that, the gluten-free diet is currently the only available and effective treatment for the condition (Green et al., 2015).

CONCLUSION

The cereals represent the major source of energy and important source of vitamins, minerals, phytochemicals, proteins, starch and fibers. The most of these are gluten source (wheat, rye, barley and some oat species). For many years studies have shown in-deep the relation of wheat storage proteins with rheological properties of dough and structural characteristics of breadmaking products such as: bread, cake and biscuits. Genetic and environmental factors continuously attract interest about their influence on specific glutenin subunits closely related to bakery quality. Celiac disease is the main health implication

associated to gluten ingestion which promotes micronutrient deficiency and atrophy of the intestinal villi. Gluten-free diet is essential to recovery the patient health. For this reason, researches have made effort to produce gluten-free food with satisfactory results when complementary ingredients are used. Recently, the health implications of gluten are not only treated as "diseases" but also are determinant on trend food market. Consumers, technologists and industries personal interests have promoted the improvement in gluten-free food label, it means advance in gluten quantification techniques and food product quality.

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Chapter 6

NON-RESPONSIVE CELIAC DISEASE

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ABSTRACT

Celiac disease is a chronic immune-mediated enteropathy triggered by the ingestion of gluten in genetically-predisposed individuals. Treatment of celiac disease is based on life-long adherence to a gluten-free dietary regimen.

Some celiac patients experience persistence or recurrence of symptoms after a period of well-being, despite an ongoing gluten-free diet. This condition, defined as non-responsive celiac disease, affects nearly one fifth of celiac patients and may be due to heterogeneous etiologies. Its occurrence warrants a correct assessment in order to optimize the management of celiac patients.

This chapter focuses on available evidence on frequency and cause of non-responsive celiac disease, and on suggested investigations for the correct assessment and management of this condition.

The initial diagnosis of celiac disease should be reconsidered. In patients with confirmed diagnosis of celiac disease, a diet compliance assessment is among the first and mandatory steps to be undertaken, to

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differentiate non-responsive celiac disease from refractory celiac disease, as the latter carries a significantly different burden of clinical implications. Further laboratory tests, breath-tests, endoscopic and histologic evaluations are warranted according to the persistent symptoms/signs in order to identify alternative or concomitant disease presenting with symptoms which overlap those of celiac disease.

Once the cause of non-responsive symptoms has been identified it should be targeted accordingly in order to achieve symptoms improvement or resolution.

INTRODUCTION

History

Celiac disease (CD) is a chronic immune-mediated enteropathy affecting the small intestine. CD is triggered, in genetically predisposed individuals, by an environmental factor represented by the ingestion of gluten, a complex of alcohol-soluble proteins of wheat, barley and rye.

CD (also called gluten sensitive enteropathy or non-tropical sprue) was described for the first time by Samuel Gee in 1888, although the description of such a chronic condition of malabsorption goes back to Aretaeus of Cappadocia in the second century B. C. [1].

The cause underlying this condition remained unknown until the Dutch pediatrician Willem Dicke described the association between the consumption of bread and cereals and recurrent diarrhea [2]. This observation was confirmed during the rationing of the Second World War, when symptoms improved with the use of foods which did not contain cereals due to the shortage of bread. After the war, with the reintroduction of bread, symptoms recurred, therefore Dicke and van de Kamer developed controlled experiments exposing children with CD to specific diets and evaluating the entity of malabsorption by determination of fecal weight and fat. Wheat, barley, rye and to a lesser extent oats caused malabsorption which proved to be reversible after exclusion of these cereals from the diet [3]. Shortly after, the responsible substance was identified in gluten, the alcohol-soluble protein component of wheat [4].

The proximal small bowel alterations characteristics of CD were described for the first time in 1954, the distinctive features being mucosal inflammation, crypt hyperplasia and villous atrophy [5].

Pathogenesis

Genetic Factors

The predisposition to CD is determined by class II molecules of the human major histocompatibility complex (HLA - Human Leukocyte Antigen Complex), in particular HLA-DQ2 and HLA-DQ8, glycoproteins of the cell membrane encoded by the HLA genes DQA1 and HLA-DQB1, located on chromosome 6p21.3 [6].

The absence of DQ2 and DQ8 makes the development of CD very unlikely [7]. However, the presence of these haplotypes is not sufficient for the development of the disease; in fact, haplotypes DQ2/DQ8 are present in about 40% of the general population while CD affects about 1% of the population in Europe with a prevalence of 1:80 - 1: 200 in Western countries. [7]. About 60-70% first-degree relatives of celiac patients are carriers of these haplotypes without ever developing the disease. The risk of developing the disease for family members with HLA-DQ2 or HLA-DQ8 is variable between 2 and 20% depending on the number of HLA-DQ2 copies and the degree of relationship [6, 8].

It is very likely that other genes, probably not HLA-associated, are implicated in the development of the disease as well. It appears that HLA explain only 30-40% of the genetic risk in the pathogenesis of CD, while the remaining 60% is attributable to non-HLA genes [9, 10], likely implicated in the immune response to gluten.

The pathogenetic mechanism is the binding of the heterodimers DQ2 and DQ8 to deamidated gliadin and tissue transglutaminase peptides with the formation of an HLA-antigen complex. This complex is recognized by CD4+ lymphocytes circulating in the intestinal mucosa, resulting in the activation of lymphocytes with production of pro-inflammatory cytokines, and consequently of histologic lesions.

Subjects who are homozygous for DQ2 have a higher concentration of HLA-DQ2 molecules on antigen presenting cells, which results in a greater binding affinity for lymphocytes compared to heterozygous, which leads to an increased inflammatory response to gluten.

The typing of HLA is useful in cases where the diagnosis is ambiguous or as a strategy for screening asymptomatic individuals belonging to high risk groups. HLA DQ2 homozygosity is associated with an increased risk of CD and enteropathy associated T-cell lymphoma (EATL) [11, 12].

Environmental Factors

The environmental factor, without which celiac disease would not develop, is represented by gliadin peptides, one of the proteic components of gluten in wheat, barley, rye and to a lesser extent oats.

It is unclear how gliadin penetrates the intestinal mucosal barrier. Physiologically there is a single layer of epithelial cells to separate the immune cells present in the submucosa by the antigens (food and microbial) present in the intestinal lumen. The possibility of a triggering event, such as a viral or bacterial infection, or a trauma has been suggested. One of the main functions of the barrier is to ensure the tolerance to common antigens, a process accomplished in part through the transformation of proteins into small peptides which are not immunogenic, in part through the destruction by apoptosis of the cells that are activated during the immune response. In CD gluten impairs the intestinal barrier [13], triggering both innate and adaptative immune responses which lead to mucosal damage.

Once penetrated into the intestinal barrier, gliadin interacts with an extracellular enzyme, the tissue transglutaminase (tTG), which represents the target autoantigen of the endomysium [14], an ubiquitous intracellular enzyme released from inflammatory cells, endothelial cells and fibroblasts in response to mechanical irritation or inflammation. Once secreted, endomysium binds to glutamine-rich proteins such as gluten proteins. The tTG is able to deamidate, in a calcium-dependent reaction, the glutamine residues of gluten, transforming them into glutamic acid. The deamidation produces a negative charge in gluten peptides, which increases their affinity for HLA-DQ2 and DQ8 molecules located on the surface of antigen presenting cells (APC), enhancing their ability to stimulate CD4 +T cells [15-17].

Gliadin peptides interact with gliadin-specific T cells that mediate tissue damage and induce the release of cytokines responsible for the damage.

In the pathogenesis of CD innate and acquired immunity are intimately connected. Both are necessary for the phenotypic expression of the disease. The adaptive response is represented by B lymphocytes, activated in plasma as a result of contact between deaminated gliadin and tissue transglutaminase; interleukin-4 released by activated $CD4^+$ T lymphocytes has the task of stimulating the expansion of self-reactive B cell clones and the consequent production of autoantibodies: anti-gliadin (AGA), anti-tissue transglutaminase (anti-tTG) and anti-endomysial antibodies (EMA) [18]. In particular, the transglutaminase type 2 (tTG2) is an ubiquitous enzyme which is present in the bowel, but also in other districts of the organism such as skin, liver, joints and thyroid; as a consequence, once the anti-tTG autoimmunity is activated, it

can extend to extraintestinal sites, so that the chain of immune reactions can become systemic, explaining many of the extra-intestinal symptoms of CD [19-21].

Epidemiology

In the ‘50s the reported prevalence of celiac disease in European countries ranged between 1:4000 and 1:8000. The picture has changed in the 70's with the acquired awareness of forms of oligosymptomatic celiac disease and the advent of sensitive and specific serological tests for dosing anti-gliadin and antiendomysial antibodies. Epidemiological studies in which these tests have been used in association with histology have reported prevalence rates of between 1:70 and 1:300 in most countries [22, 23].

Population studies have suggested that diagnosed cases of celiac disease represent the tip of the iceberg. One of the major screening programs on celiac disease was performed on 17,201 school children, aged between 6 and 15 years, enrolled in various Italian regions [24]. The prevalence of celiac disease was equal to 1:184 and the ratio between undiagnosed subjects and diagnosed subjects was 7:1.

A comparable prevalence (1:256) was reported in a screening study of 1,866 blood donors in Sweden [25].

A 2003 US study in which 13,145 individuals were screened [26] showed that in at-risk groups, the prevalence of celiac disease was 1:22 in first-degree relatives (4.5%), 1:39 in second-degree relatives (2.6%), 1:56 in symptomatic subjects (1,8%) compared to 1:133 in the general population (0.75%).

Other groups at increased risk of developing celiac disease are individuals with Down and Turner syndrome, type 1 diabetes, autoimmune thyroid disease, with prevalence rates ranging from 5 to 10%, [27].

Clinical Presentation

Gastrointestinal Symptoms

Patients may present with typical symptoms such as diarrhea and steatorrhea associated with signs of malabsorption, such as growth failure in children, weight loss, severe anemia, neurological symptoms of B vitamins deficiency and osteopenia by vitamin D and calcium deficiency.

However, more and more patients have milder or atypical symptoms or are asymptomatic [28].

Subclinical Disease

The spread of the serological screening has shown that celiac disease can present with minor, non-specific clinical symptoms such as fatigue, iron deficiency, ipertransaminesemia or can be completely asymptomatic [29].

This explains the high percentage of patients who remain undiagnosed. Many of the patients without specific symptoms receive the diagnosis of celiac disease in the course of screening programs or as incidental finding during investigations carried out for other reasons.

Studies have documented that the prevalence of autoimmune diseases (eg. diabetes mellitus type I, vasculitis, autoimmune thyroiditis) correlates with the duration of undiagnosed celiac disease and can exceed 30% in patients diagnosed after the age of 20 year [30].

Oligosymptomatic celiac patients may have nutritional deficits. In one study 31% of patients with subclinical disease showed signs of malnutrition, compared to 67% patients with typical symptoms [31].

Extra-Intestinal Symptoms

In some patients extra-intestinal symptoms are the symptoms of onset of the disease. The most common are infertility, rheumatologic diseases, osteopenia and osteoporosis and neuropsychiatric symptoms [32].

Iron deficiency - Celiac disease is a very frequent cause of iron-deficiency anemia [33-35]. A study on 93 patients referred for evaluation of iron-deficiency anemia showed that in 11 patients (12%) a duodenal biopsy was compatible with celiac disease [36].

Similar results were reported in another paper in which 6% of 85 patients with iron-deficiency anemia resulted affected by celiac disease [33].

The incidence was 20% in the subgroup of patients who did not respond to martial supplementation.

Metabolic bone disease - Metabolic bone disease is common in celiac patients and may be present in patients without gastrointestinal symptoms [37-39]. A study compared bone mineral density and the prevalence of osteopenia and osteoporosis in 77 celiac patients versus 157 controls [40]. Celiac patients had a significantly reduced bone density at both the lumbar spine and femoral neck compared with controls (-6 and -5% respectively). They also had a significantly higher risk of developing osteoporosis at the lumbar spine (26

versus 5%). These patients had secondary hyperparathyroidism probably due to vitamin D deficiency [41].

Dermatitis herpetiformis - The classic clinical manifestation is the development of intensely itchy papules and vesicles with "herpetiformis" distribution. The most frequently affected body areas are the elbows, the dorsal region of the forearms, knees, scalp, back, and buttocks. The face and groin are the least frequently affected sites. The diagnosis is confirmed histologically by the finding of granular deposits of IgA along the basement membrane [42].

The anti-tTG antibodies are elevated in these patients. Autoantibodies are mainly directed against the epidermal transglutaminase, which has a high sequence homology with tTG [43].

In a study on a Finnish population celiac disease was diagnosed in 398 of 147,000 individuals (prevalence 1:369), of which 24% were suffering from dermatitis herpetiformis [44]. There are no precise figures on the proportion of patients with dermatitis herpetiformis who are also suffering from celiac disease, but it is probably a ratio around 85% when the diagnosis of celiac disease is made histologically [45].

Dermatitis herpetiformis and celiac disease are associated to the same heterodimers HLA-DQ α / β [46-48].

Dermatitis herpetiformis responds to the gluten-free diet (GFD) [49]. However, the improvement may take a long time (months or years) in the absence of additional, targeted treatment [50-51].

IgA deficiency - a condition which is frequently associated with celiac disease is selective IgA deficiency [52]. Screening programs have documented the presence of celiac disease in up to 8% patients with selective IgA deficiency [53]. On the other hand, IgA deficiency is found in 1-2% celiac patients [54]. This condition is often clinically not relevant but must be taken into consideration as it may cause false negative serological test results. In patients with IgA deficiency screening must be done by dosage of anti-tTG IgG.

Liver disease - Celiac disease is associated with chronic elevated transaminases [55, 56]). A meta-analysis described that patients with cryptogenic hypertransaminasemia have a positive serology for celiac disease in 6% patients and a histological diagnosis of celiac disease in 4% [57]. With the establishment of a GFD a normalization of ALT was observed in 63-90% of patients within one year. Celiac disease has also been associated with advanced liver disease [58-60]. In a study on patients with severe liver disease

(due to congenital hepatic fibrosis, steatosis and massive hepatitis NOS) and undiagnosed celiac disease, a significant improvement of liver function was documented following the introduction of GFD [61]. Celiac disease was diagnosed in 8 of 185 adult patients undergoing liver transplantation for chronic progressive liver diseases such as primary biliary cirrhosis (PBC), autoimmune hepatitis, primary sclerosing cholangitis (PSC) and congenital hepatic fibrosis.

Inflammatory Bowel Disease (IBD) - Various case series have shown an association between CD and IBD, most frequently with ulcerative colitis than with Crohn's disease [62-63]. In a case-control study, the risk of IBD in celiac patients was increased by 10 times, while the risk of celiac disease in patients with IBD was similar to that of controls [64].

Two independent studies have documented the presence of a common polymorphism of pro-inflammatory IL-23 receptor gene in celiac disease and ulcerative colitis [65, 66]. First-degree relatives of celiac patients seem to have a 5 times higher risk of developing ulcerative colitis than the general population [67]. In a case-control study of 51 patients with concurrent IBD and celiac disease and 102 controls with IBD only, patients with both diagnosis were more likely to have a pancolitis than patients with ulcerative colitis only (OR 3.3, 95% CI 1.05-21.5) [68].

Gynecological and reproductive problems - women with active celiac disease may have delayed menarche, premature menopause, secondary amenorrhea, recurrent fetal loss, infertility, preterm birth and low birth weight infants [69-77].

One study compared 588 women with reproductive problems with 305 women with a normal obstetric history [72]. None of the women in the study had typical gastrointestinal symptoms of celiac disease. Women with reproductive problems were more likely to have a positive serology for celiac disease (IgA anti-tTG). Since in these patients a histologic confirmation was not made it is likely that this figure is overstated.

A cohort study on 211 infants showed a 3-fold increased risk of growth retardation in infants born from mothers with unrecognized celiac disease than controls [78].

Celiac disease has also been described in association with male infertility, characterized by abnormalities in motility and morphology of spermatozoa and androgen resistance [79, 80].

Diagnosis

Serology

Serological tests used in the diagnosis of celiac disease include:

IgA anti-endomysium (EMA IgA)
IgA anti-tissue transglutaminase (tTG IgA)
IgG anti-tissue transglutaminase (tTG IgG)
IgA anti-deamidated gliadin peptide (DGP IgA)
IgG anti-deamidated gliadin peptide (DGP IgG)

IgA EMA, IgA tTG, DGP IgA and IgG levels DGP declines with the gluten-free diet and can therefore be used as non-invasive tests for diet response monitoring.

Serological tests for celiac disease can be divided into two groups according to their target antigens: endomysial and gliadin antibody tests [81].

Anti-Gliadin Antibodies (AGA) were the first serological marker to be used in clinical practice. Currently they have been put aside because of low sensitivity and specificity. The false positivity of AGA IgG is a fairly common phenomenon as these antibodies may test positive in other gastrointestinal disorders; they are still used in children under the age of two years, as they can become positive before EMA. AGA are assayed by ELISA (enzyme-linked immuno-sorbent assay) using purified gliadin as antigen;

Second generation AGA tests (anti-deamidated gliadin peptide [DGP]) use synthetic gliadin peptides that mimic the gliadin sequences modified by tTG and show high diagnostic accuracy [82, 83].

Anti Endomysial Antibodies (EMA) are detectable by indirect immunofluorescence (IFI) [84, 85]. Frozen sections of monkey esophagus have been initially used for the assay. Currently many laboratories use human umbilical cord sections [86, 87]. The test result is simply reported as positive or negative, since even low titers of anti-endomysial IgA serum are specific for celiac disease. Their accuracy is high: 99.8% for specificity and 95% for sensitivity [81, 88-91]. In a screening study of 4,615 adults antibodies anti-endomysial IgA had a positive predictive value of 100 percent; positive predictive values for anti-gliadin antibodies IgG and IgA were only 2% and 12% respectively, due to their lower specificity [92].

Anti-tissue transglutaminase - The antigen against which EMA are directed is a tissue transglutaminase (tTG) [93]. The anti-tTG antibodies are highly sensitive and specific for the diagnosis of celiac disease [94-96]. They

are assayed by enzyme immunoassay and radioimmunoassay methods using a recombinant human enzyme as substrate [97]. They have high sensitivity (95-98%), and specificity (94-95%); in comparison to EMA they have the advantages of having a greater ease of technical implementation, therefore being less operator dependent, and a low cost. In a descriptive work, anti-tTG antibodies were present in 98 percent of patients with celiac disease diagnosed histologically compared to 5 percent of controls [98]. In another study including 136 patients with celiac disease and 207 controls, the sensitivity and specificity of anti-tTG antibodies were 95 and 94% [99] respectively.

ELISA for the detection of anti-tTG IgA antibodies is now widely available, easy to use and cheap in comparison to immunofluorescence used to detect EMA IgA.

The antibody positivity is not in itself diagnostic, and must be correlated with the results of the intestinal biopsy, allowing to discriminate overt celiac disease from the latent or potential forms. This test is used to screen patients at increased risk such as first-degree relatives of celiac subjects or patients with diseases presenting with a high association with celiac disease such as insulin dependent diabetes mellitus, autoimmune thyroiditis, Down syndrome, Turner syndrome. Moreover, these antibodies are used in the monitoring of celiac patients on a GFD.

IgA anti-tTG are the test of choice for the detection of celiac disease in subjects older than two years.

When there is a high probability of CD (> 5 percent), the measurement of total IgA should also be carried out in order to avoid a false negative due to selective IgA deficiency. An alternative approach in subjects with a high probability of celiac disease is to search for both IgA and IgG anti-deamidated gliadin IgG (DGP).

The currently accepted criteria for the diagnosis of celiac disease require the positivity of anti-tTG antibodies, a small intestinal histology compatible with CD and documented clinical improvement with GFD [100]. Duodenal biopsy can be avoided in children and adolescents who have a strong clinical suspicion and high titer of anti-tTG, with levels exceeding 10 times the normal limit.

Histology

Patients with a positive serology and patients with a high probability of celiac disease (>5%) regardless of serology should undergo endoscopy of the

upper gastrointestinal tract and small intestine biopsy to confirm the diagnosis of celiac disease. The only exceptions are children with a high titer positive serology.

The currently accepted standards for duodenal sampling include at least 4 biopsies from second or third duodenum and at least one biopsy from the bulb (at 9 or 12 o'clock) [6, 100-103]. For a long time, biopsies in the duodenal bulb were avoided because of the perceived risk of false positives due to acid-induced damage and technical problems. However, more recent evidence showed that not only the atrophy is present both in the second portion and the bulb, but in some patients, particularly younger ones, the histological alterations, which in CD may have a "patchy" distribution, are present only in the bulb [104].

Macroscopically, alterations that can be seen during endoscopy are: reduction or disappearance of the folds of Kerckring, indentations of duodenal folds, nodular appearance of the mucosa, mosaic pattern and visualization of the submucosal vascular pattern, which is normally not visible at duodenal level.

The histology in celiac disease is most commonly classified using the Marsh-Oberhuber classification [105]:

- Marsh stage 0: normal intestinal mucosa. The villi have a normal elongated shape with a ratio between the height of the villi and crypts of 3/1 or greater; a biopsy is considered normal if it contains at least 4 normal villi. Enterocytes are normal in number and size; the intraepithelial lymphocytic infiltration is normal within the range of 25 lymphocytes per 100 epithelial cells; in the lamina propria there is no evidence of inflammatory infiltrate.
- Marsh grade 1: also called "infiltrative" phase. The villi are architecturally and morphologically normal (normal villous/crypt ratio of 3/1), the crypts are normal, but there is an increase in the number of intraepithelial lymphocytes, above 25 IEL/100 epithelial cells (EC). Alterations of this type are generally found in celiac patients on a GFD [106]. This degree of injury can be frequently found in people with dermatitis herpetiformis or first-degree relatives of celiac patients. Despite the high risk at this stage we cannot make a diagnosis of CD at this stage, so these patients should undergo a careful follow-up in order to monitor the possible development of the disease.

A lymphocytic infiltration of the intestinal epithelium in the absence of villous atrophy is not specific for celiac disease and must be considered in the differential diagnosis of other causes [107]. In fact, factors other than gluten exposure may account for Marsh 1 lesions including medications such as nonsteroidal anti-inflammatory drugs (NSAIDs) and proton-pump inhibitors (PPI), *Helicobacter pylori*-related gastritis and other infective enteritis.

- Marsh grade 2: also known as "hyperplastic" phase. The villi are architecturally still within normal limits; at staining with hematoxylin-eosin there is a hyperplasia of the glandular elements with regenerative aspects highlighted by an increased number of mitosis. The intraepithelial lymphocytes are always above 25 IEL/100 EC.
- Marsh grade 3: also called "atrophic" phase. Enterocytes surface have a reduced height, with an irregular brush-border and possibly cytoplasmic vacuoles; intraepithelial lymphocytes are always above 25 IEL/100 EC; there is associated crypt hyperplasia of variable degrees; Oberhuber suggested a further subdivision on the basis of the degree of villous atrophy.

Type 3a: mild villous atrophy, IELS > 25/100EC and crypt hyperplasia.
Type 3b: moderate villous atrophy, IELS > 25/100EC and crypt hyperplasia.
Type 3c: severe villous atrophy, IELS > 25/100EC and crypt hyperplasia.

- Marsh Grade 4: This picture is extremely rare and is characterized by total villous atrophy, with normal crypts and normal IELS count. This type of lesion is characteristic of Enteropathy Associated T-cell Lymphoma (EATL).

There are a number of diseases that can cause villous atrophy in non-celiac patients, including Crohn's disease, infectious enteritis, duodenitis, immune deficiencies, and some medications such as NSAIDs and olmesartan [108-109, 167-169].

Classification

The clinical manifestation of CD shows an extreme variability of symptoms in relation to age, amount of gluten ingested with the diet, duration and extent of the disease. Celiac disease can be divided into different clinical forms:

Overt or "classic" celiac disease - the typical onset symptoms are gastrointestinal, with diarrhea and malabsorption. It is most common in children, occurring a short time after the introduction of gluten in the diet. Other associated symptoms can be pain and abdominal distension, flatulence, vomiting, heartburn, loss of appetite and weight loss, growth retardation in children. It presents with villous atrophy, symptoms of malabsorption or other signs of malnutrition [110]; the resolution of clinical manifestations and histological damage usually occur within a few weeks or months after the introduction of a GFD. The severity of duodenal histological changes does not correlate with the severity of clinical manifestations [111].

Atypical celiac disease – It is the most common form in adults and adolescents. It presents with milder symptoms and prevalence of extra-intestinal symptoms. Usually diarrhea is absent and the clinical manifestations are secondary to malabsorption including: short stature, iron deficiency anemia and/or folic acid or vitamin B12 deficiency, which are often not responsive to oral supplementation; juvenile osteoporosis in premenopausal women, hyperphosphatemia and hypocalcemia due to bone metabolism disorders [112]. Dysplasia of the tooth enamel may also be present, ranging from discoloration to the presence of pitting, and up to total loss of enamel; infertility, menstrual irregularities and recurrent fetal loss are also common (the risk of miscarriage is 9 times higher in women with untreated celiac disease than healthy women and a GFD appears to reduce the risk of abortion in CD patients) [113]. Other symptoms of the atypical form include recurrent abdominal pain, recurrent aphthous ulcerations due to vitamin deficiencies, delayed puberty, constipation, idiopathic hypertransaminasemia, vitamin K deficiency, alopecia [114]. There may also be neurological symptoms such as epilepsy, ataxia, mood disorders [115].

These patients have serology and duodenal histology comparable to those of patients with "classic" CD.

Silent celiac disease - Patients with positive serology and overt intestinal damage in the absence of any clinical symptoms reported. Serology and intestinal damage resolve with a gluten-free diet. In such patients, the diagnosis of celiac disease is often incidental. The existence of these clinically

silent forms was demonstrated through screening of asymptomatic first-degree relatives of celiac patients.

In reality this form is frequently asymptomatic in appearance only, and may present with atypical and mild symptoms, which however can be recognizable at a careful clinical monitoring: psychophysical discomfort (deflected mood, irritability, fatigue), iron deficiency with or without anemia, low bone mineral density [116].

Patients often realize that they had minor symptoms (e.g., fatigue) only after the introduction of a gluten-free diet which often leads to a significant improvement of their general well-being.

Latent celiac disease - Patients with normal mucosa, positive serology and asymptomatic or with mild symptoms during free diet [117]. Two latent CD variants have been identified:

1) Celiac disease which was present in the past, usually in childhood; the patient recovered completely with a gluten-free diet and celiac disease has remained in remission even after the re-introduction of a free diet. About 20% of these patients continue to have latent disease (asymptomatic with normal villous architecture) in adulthood, while in the remaining 80% variable degrees of villous atrophy recur [118]. Latency can be transient, and therefore these patients require a regular follow-up.
2) The finding of normal mucosa is demonstrated at an earlier stage of the development of the lesions and the disease develops later.

Therapy

The treatment of celiac disease is based on life-long adherence to a GFD; this regimen result in remission of clinical symptoms and histological alterations in the majority of patients.

A GFD is recommended for patients with “classic”, atypical and silent CD. In patients with latent celiac disease there is currently no indication to follow a GFD, but there is indication to clinical monitoring and possible repetition of duodenal sampling.

There is a high inter-patient variability in the ability to tolerate gluten: while some patients are extremely sensitive to even small amounts of gluten, other patients can tolerate small re-introduction of this substance in the diet after reaching remission. Despite this variability, strong evidence support the

indication to a strict adherence to a GFD in patients with celiac disease, irrespectively of clinical symptoms. Even patients who do not experience any symptoms or signs should not underestimate the possible presence of occult micronutrient deficiencies, which can lead with time to clinical consequences, such as loss of bone mass due to deficiency of vitamin D [38]. Numerous studies have also suggested an increase in overall mortality and an increased risk of developing cancer (e.g., lymphoproliferative disorders and gastrointestinal cancer) in patients with CD compared to the general population [119].

Two studies concluded that the likelihood that patients with CD develop other associated autoimmune disorders (such as type 1 diabetes, connective tissue disease, thyroid disease) seems to be related to the duration of exposure to gluten [30, 120].

Mothers who have undiagnosed CD appear to be at increased risk of having infants with low birth weight and premature births than mothers with known CD following a GFD [78, 121].

Adherence to a GFD in celiac subjects is estimated to be variable between 40 and 90% [122-124]. The cost, the limited availability and poor palatability of gluten free foods, as well as meals outside home can decrease adherence. It is estimated that, in reality, a complete non-adherence to the GFD is infrequent, with a rate of less than 5% of subjects in the majority of studies (range 0-32%) [124].

Foods are considered gluten-free if they naturally contain a level of such substance less than 20 mg/kg in total or are processed foods with level less than 100 mg/kg.

A study that evaluated the inadvertent intake of gluten (from wheat contaminants) in 76 patients who followed a GFD, estimated that the contamination of gluten up to 100 parts per million (up to a total of 30 mg per day) does not cause histological lesions [125].

It is interesting to note that 13 of the 59 naturally gluten-free products, and 11 of 24 processed gluten-free products contain 20 to 200 mg/kg of gluten.

The most common causes of exposure to gluten are represented by high frequency of meals eaten outside home or inadequate knowledge of foods to avoid [124]. When suspected, the evaluation of exposure to gluten should be assessed through a detailed dietary history, the use of a nutritional diary and a consultation with an expert nutritionist in order to identify possible cause of inadvertent contamination and to implement adequate dietary changes. The identification of possible sources of contamination, the understanding of all aspects of the GFD, regular follow-up at a referral center for CD and joining a

group for CD patients support seem to be all predictors of adherence to the diet. [124, 126].

Monitoring

The rapidity of the response to the GFD is variable.

Generally an improvement in clinical condition is perceived after days or weeks from the beginning of the diet. About 70% of patients have a clear clinical improvement within two weeks [127]. The achievement of a histological recovery usually requires about 6-12 months in the majority of CD patients [106].

The reason why the symptomatic improvement is much faster than the histological recovery is not completely understood. Some authors reported as a possible explanation that the distal small bowel is generally less severely damaged, and therefore recovers faster, than the duodenum where the biopsies are performed, which is typically affected more severely because of its greater exposure to gluten [128].

In less than 25% of celiac patients histologic recovery is obtained after a period equal or exceeding one year from the beginning of the diet [129]; it has been described that in a small percentage of patients complete recovery takes up to 5 years; this subgroup has been called “slow-responders” [130].

Serology - IgA anti-tTG or IgA (or IgG) anti-DGP are used to monitor the response to the GFD [107]. The baseline antibody level at diagnosis must be recorded and used as a mean of comparison. The effective exclusion of gluten from the diet results in a gradual decline in the levels of IgA anti-tTG IgA and serum anti-DGP (which have a half-life of six to eight weeks).

With a good dietary compliance, normalization of serology is usually achieved within 3-12 months, depending on the antibodies titers before treatment. Normal levels of IgA anti-tTG are not indicative of histological recovery [96].

Persistently high levels of antibodies reflect continuous exposure to significant amounts of gluten in the diet; on the contrary, antibody levels are not a sensitive indicator of occasional or minor dietary transgressions [131].

Small bowel biopsy - The need for a follow-up biopsy in patients with clinical improvement on a GFD has long been debated. Endoscopy of the upper gastrointestinal tract with duodenal biopsy sampling should be performed in patients with a certain diagnosis of CD that do not have a

substantial clinical response to a GFD or who have recurrence of symptoms despite a GFD [107].

Gluten rechallenge - the gluten reintroduction has been considered unnecessary in patients with symptomatic, serologic and histologic improvement [100].

The reintroduction of gluten is useful only in cases of uncertain diagnosis, for example if no initial biopsy was performed at first diagnosis, in case of inadequate biopsy sampling or in communities with a high risk of other enteropathies [100].

Gluten reintroduction should be carried out for at least 8 weeks before obtaining duodenal biopsy sampling with the understanding, however, that the relapse may take up to several years. A rare condition has been reported following gluten challenge, presenting with fulminant diarrhea with dehydration, acidosis, and other metabolic disorders. This condition, known as "gliadin shock", is treated with corticosteroids [132].

NON-RESPONSIVE CELIAC DISEASE

Definition

Non responsive celiac disease (NRCD) is defined as the failure of clinical and/or histological improvement with a GFD [133-135].

NRCD can be further classified into primary, when a patient on a GFD never achieved a complete remission following the initial diagnosis of CD, or secondary, when a patient with an initial response to the diet experience the relapse of symptoms despite a continue GFD [134-135].

Frequency and Causes

The exact prevalence of NRCD remains to be determined, although retrospective studies have shown that NRCD can occur in 5-30% of patients with CD [127, 136].

One of the first reports of NRCD dates back to 1967, when Pink et al. described a group of 54 celiac patients followed for a period ranging between 6 months and 14 years; a poor response to the GFD was reported in 30% of patients [127]. Over the years the reported prevalences have been variable in

different studies, possibly depending on the type of centre (secondary, tertiary) and therefore patient populations studied.

One of the most recent studies targeting the issue reported a prevalence of NRCD of 21.8% (70/320 patients, 10 males and 60 females) in CD patients on a long-term GFD (>12 months); thirty-six patients (52%) complained of intestinal symptoms, the most common being diarrhea and bloating, 17 patients (24%) reported the persistence of extra-intestinal symptoms and 17 patients (24%) complained of both intestinal and extra-intestinal symptoms [137].

Causes of NRCD reported in literature include: incorrect initial diagnosis of CD, persistent voluntary or inadvertent ingestion of gluten, concurrent conditions that manifest with symptoms/signs resembling those of CD and refractory celiac disease [133-141].

Initial Misdiagnosis of CD

In a retrospective case series of 55 patients referred to a tertiary center, in 6 patients (11%) the diagnosis of CD was disproved [138]. In another study on 112 NRCD patients referred to a tertiary care center, in 12 patients the diagnosis of celiac disease was disproved; these patients underwent a gluten challenge followed by a negative duodenal histology [133].

In a retrospective study on 603 patients followed over a period of five years, of which 113 were classified as NRCD, the diagnosis of CD was disproved in 14/113 (12.4%) after the revision of the serological and histological data related to first diagnosis [134].

A recent study which analyzed the causes for symptoms persistence in 56 NRCD patient in a tertiary centre reported that in 3/56 patients the evidence regarding the initial CD diagnosis was unconvincing [137]. Assessment of HLA status showed that all 3 patients were HLA-DQ2/8 negative. These patients underwent gluten challenge and duodenal sampling with histology, which showed no mucosal alterations, thereby disproving CD diagnosis. Eighteen out of the 70 (26%) initially identified NRCD patients had received the initial CD diagnosis in other institutions while in 52/70 (74%) the diagnosis was made at the institution of the Authors [137].

Gluten Contamination

In a retrospective case series on 55 patients, after exclusion of initial misdiagnosis (6 patients), 51% of the remaining patients (25 of 49), referred to as NRCD, had a persistent voluntary or accidental ingestion of gluten, confirming gluten contamination is the leading cause for symptoms persistence. At the interview for the assessment of adherence to the diet, these 25 patients believed they strictly observed the GFD [138].

A retrospective study conducted in a tertiary referral center on 603 patients over a period of five years reported that 113 patients (18.7%) could be classified as NRCD. The authors reported gluten exposure as the commonest cause for symptom persistence in 36% of patients [134].

In 35 of 99 patients (36%), of which 9 males and 26 females, with confirmed CD diagnosis and persistent symptoms during GFD, accidental gluten ingestion was identified [133]. Twenty-two of these 35 patients (62.9%) had a primary form of NRCD (4/22 males, 18/22 females). In the group of 35 patients with evidence of gluten contamination, the most frequent persistent symptoms were diarrhea (55%), abdominal pain (35%) and weight loss (15%). An increase in anti-tTG IgA titers (> 20 U / ml) proved predictive of exposure to gluten (OR 11.3, 95% CI, 3.7 to 34.4); during the course of the study this dosage was repeated in 28 of 35 patients (80%), of whom 22 (78%) had levels of anti-tTG IgA persistently higher than 20 U / ml [133].

More recently, the finding of increased levels of anti-DGP IgG antibodies in CD patients on strict GFD has been proven to effectively identify patients with NRCD who have persistent villous atrophy. The Authors suggest that anti-DGP IgG antibodies can be considered a useful tool to monitor mucosal damage and histological improvement in CD patients on a GFD [142].

In a tertiary care centre study on 112 NRCD patients, after the exclusion of 12 uncorrect diagnosis, in 45 of 100 (45%) patients, gluten ingestion was identified, which was accidental in 24 patients (53.3%) and voluntary in 21 patients (46.6%) [133]. Thirty-seven of 45 patients (82.2%), had a repeated endoscopy with duodenal biopsy sampling. Histology showed mucosal damage (Marsh IIIa-IIIc) in 33/37 cases (89%). All the 45 patients in whom the ingestion of gluten was demonstrated reported an improvement of symptoms following dietary advice and strict adherence to the GFD; the most common form of accidental contamination occurred with malted breakfast cereals, beer, cooking sauces, pizza and cookies [133].

In 75.7% patients (28 of 37) undergoing a further endoscopy with duodenal biopsies after a strict adherence to the GFD, 27 (96.4%) showed

histological improvement; the only patient who did not have a histological improvement admitted the possibility of contamination [133].

A recent prospective study reported that in 7/56 NRCD patients, all with EMA positivity, evidence of inadvertent and/or voluntary gluten ingestion could be supposed based on the reported GFD compliance and the presence of potential causes of exposure to gluten [137]. These patients received dietary advice by trained gastroenterologists and attended a regular follow-up. Six months later, a further antibody evaluation showed that 5 out of 7 patients became EMA negative. The 2 patients who remained EMA positive underwent EGDS and biopsy sampling, which showed CD-related histological alteration (1 Marsh 3A and 1 Marsh 2). Both patients reported persistent ingestion of gluten despite the dietary advice provided by gastroenterologists. Another 5 patients, which were EMA negative and underwent upper endoscopy with biopsy to clarify NRCD cause, had evidence of villous atrophy and reported episodic (3 patients) or continued (2 patients) gluten intake. Overall, in patients with confirmed diagnosis of CD, exposure to dietary gluten was the main cause for persistence of symptoms/signs, and consistently, after dietary modification, symptoms resolved in 63% of the patients at later time points during follow-up [133].

Associated Conditions Accounting for Symptoms

Multiple conditions have been reported to account for symptoms in patients with CD, including irritable bowel syndrome [IBS], microscopic colitis, giardiasis and other intestinal infections, lactose intolerance, inflammatory bowel disease, small intestinal bacterial overgrowth, thyroid impairment, common variable immunodeficiency, pancreatic insufficiency, agammaglobulinemia [133-141].

In a retrospective study on 603 patients over a period of five years which identified 113 patients as NRCD, IBS was the most common associated condition accounting for symptoms persistence (22%). Other etiologies for reported symptoms were documented, including irritable bowel syndrome (22%), refractory celiac disease (10%), lactose intolerance (8%), and microscopic colitis (8%) [134]. Seventy-four (65%) of 113 patients had received the initial diagnosis of CD in other institutions, and 39/113 (35%) were diagnosed at the institution of the study authors. The mean duration of symptoms was 15.3 months (range 1-138 months). The only statistically significant difference between NRCD and diet-responsive CD in this cohort of

patients was having or not been diagnosed in a referral center (27.6% responsive CD versus 64.6% NRCD, p <0.001) [134]. This finding, confirmed by subsequent studies [137], shows that despite there is wide consensus on diagnostic criteria for CD, errors can still be made in clinical practice.

A tendency to a higher prevalence of psychiatric disorders was observed among NRCD compared to patients with responsive CD, not reaching statistical significance (31.9% vs 23.7%, p = 0.09). No difference in terms of sex, initial anti-tTG IgA antibody levels, mean age at diagnosis or coexistence of other autoimmune diseases was observed between the two groups [133].

A case-control study on 54 male CD patients reported low testosterone as a cause which may mimic NRCD symptoms [143]. Patients presented more commonly with fatigue and osteoporosis. Although low testosterone occurs in CD patients at a similar rate as the general population, a testosterone panel testing should be performed in male CD patients presenting with such symptoms, before pursuing more invasive investigations.

Microscopic colitis (including collagenous and lymphocytic colitis) has been described in association with CD. Microscopic colitis was reported in 4% of patients with CD [139].

A prospective study on 56 NRCD patients reported various concomitant diagnosis to account for symptoms: one patient reporting fatigue and arthralgias as persistent symptoms received a diagnosis of fibromyalgia; three patients with persistent diarrhea were found to be lactose intolerant and experienced resolution of symptom following a lactose-free diet; two patients had non-erosive gastro-esophageal reflux disease (GERD), which promptly responded to treatment with proton pump inhibitors; nine patients were diagnosed with irritable bowel syndrome (IBS), among which 5 complained of constipation with documented benefit from fiber supplementation, while the remaining 4 had a diarrhea-predominant IBS, which improved following treatment with probiotics [137].

In patients with CD with persistent diarrhea and in whom abnormalities of exocrine pancreatic function are documented, with low levels of fecal elastase, clinical benefit has been reported following pancreatic enzymes supplementation [140, 141].

Refractory Celiac Disease

Refractory celiac disease (RCD), or refractory sprue is a rare and potentially serious condition defined by the persistence of villous atrophy

despite strict adherence to a GFD and no evidence of other disease, including lymphoma, to justify such clinical picture [144].

The exact incidence of RCD is not known, but referral centers have estimated its prevalence to be around 8-18%, with a slightly lower incidence rate when the initial diagnosis of CD has been made in a referral center [144, 145].

Refractory sprue has been classified into two immunological categories, defined as type I and type II, depending on the population of intra-epithelial T cells [144, 146]:

- In type I RCD the population of intraepithelial lymphocytes is normal and polyclonal.
- In type II RCD there is an aberrant intraepithelial lymphocyte population on the basis of the analysis of T cell receptor clonality and immunophenotyping. Patients with type II RCD have a population of intraepithelial lymphocytes with an aberrant phenotype (IELS $CD103^+$, intracellular $CD3^+$, $CD4^-$, $CD8^-$, superficial $CD3^-$) with a clonal rearrangement of the gamma chain of the T-cell receptor (TCR)-γ, detectable through polymerase chain reaction (PCR) on biopsy samples. Type II RCD patients show poor response to treatment, a high rate of progression to lymphoma (approximately 50% of patients in a period of 2 years of follow-up), and a higher mortality compared to type I (41% vs. 14% in 2 years; 42-56% vs 4-7% at 5 years) and to patients with responsive CD [147].

Type II RCD can progress to enteropathy associated T-cell lymphoma (EATL) [145]. The diagnosis is made on the basis of histology; radiologic studies including computed tomography (CT), magnetic resonance (MRI) and positron emission tomography (18F-FDG PET) can be helpful in the identification of suspicious areas [148, 149].

The differentiation between type I and type II RCD is important both for management and prognosis [107]. Patients with type I RCD have a less severe clinical presentation and a much better prognosis than patients with type II RCD [150, 151]. Moreover, type I does not seem to evolve into type II. A descriptive study compared the outcomes of 41 patients with type I RCD and 50 patients with type II [150]. The 5-year survival was higher in type I group (96 versus 58%). Most of the deaths were due to the development of EATL (which developed in half of the patients during follow-up). No patients with

type I RCD developed type II disease over an average period of five years of follow-up [150].

A subgroup of patients with RCD develop the deposition of a subepithelial collagen band, a condition known as “collagenous sprue” [152].

Persistent symptoms that seem indicative of malabsorption in patients with CD on a prolonged GFD should raise the suspicion of RCD. In this scenario, the biopsy samples should be analyzed for IELS phenotyping and PCR for the study of TCR clonality.

The appearance of other symptoms such as abdominal pain, night sweats, fever, gastrointestinal bleeding or sub-occlusive symptoms could be suggestive of evolution in ulcerative jejunitis, EATL or adenocarcinoma of the small intestine [145].

RCD can be classified into a primary form, when symptoms and atrophy of the villi persist despite the strict adherence to a GFD for a period longer than one year after diagnosis, and a secondary form, if symptoms and villous atrophy recur after an initial period of improvement during GFD for at least one year [144].

The cause of refractory sprue is currently unknown; one theory suggests that some patients with this condition develop sensitivity towards different food components other than gluten [153]. Patients with refractory sprue should be closely monitored and receive nutritional support, including parenteral nutrition, if necessary.

The treatment of refractory sprue is based on immunosuppression, which has been traditionally achieved with glucocorticoids.

The dose of glucocorticoids required has not been established and patients response to steroid can be variable. In patients with type II RCD steroids are associated with immunomodulators such as thiopurines. More recently, a variety of other immunosuppressant agents including anti-TNF alpha have been used to treat RCD in small studies [154].

However, experience with alternative immunosuppressive therapy in patients requiring high doses of glucocorticoids is limited [155-158]. Clinical and histological improvement after treatment with elemental diet in patients with type I RCD has been described [159].

Another report described the induction of remission in a patient with type II RCD treated with Alemtuzumab, an anti-CD52 monoclonal antibody used to treat chronic lymphocytic leukemia [160]; However, the drug has not proven effective in another study [161]. Cladribine (a synthetic purine nucleoside with cytotoxic activity) has been associated with clinical and histological improvement in 6/17 patients with type II RCD [162].

In the previously cited study by Dewar et al. [133] 9 of 55 NRCD patients (16.4%) were diagnosed with RCD. Of these 9 patients, 2 were type I RCD and 7 were type II RCD (3 patients with ulcerative jejunitis, 4 patients with EATL). Of the 4 patients with EATL, two had a survival of less than 1 year after diagnosis and two were alive at the end of the study (one treated with immunosuppressive therapy, the other underwent successful resective surgery). In summary, in this cohort of patients with CD from the United Kingdom, 3 of 9 patients with RCD died during the follow-up period [133]. In the study by Leffler et al, 10 of 99 NRCD patients (10.1%, 6 males and 4 females), received a diagnosis of RCD. Fifty percent of patients were primary RCD (3/6 males, 2/4 females) [134]. The clinical presentation was characterized by weight loss, diarrhea and abdominal pain in 90%, 70% and 80% of cases respectively. Weight loss seems to be predictive of RCD with a 31.1 OR (95% CI, 5.9 to 163.1) [134].

A retrospective study carried out in a tertiary referral centre described 17 GFD-adherent NRCD patients, 6 of which were classified as having type I RCD. These patients underwent a modified dietary regimen that aimed to eliminate any possible sources of gluten cross-contamination in an already strict GFD for a period of 3-6 months. Fourteen out of 17 patients (82%) responded to the modified diet. Of the 6 patients meeting the criteria for type I RCD prior to the diet, 5 (83%) were asymptomatic after the diet, and no longer meeting the criteria for RCD. Of the 14 patients who responded to the modified diet, 11 (79%) successfully returned to a traditional GFD without recurrence of symptoms. The authors concluded that a limited period of a modified, unprocessed gluten elimination diet, may be an effective therapeutic option for GFD-adherent NRCD patients and that the response to this diet identifies a subgroup of patients, which can be misclassified as having type I RCD, that is not truly refractory to dietary treatment [163].

Management

It important to distinguish RCD from NRCD due to other causes, as there is a considerable difference in the morbidity and mortality of these conditions mainly due to the development of complications that often accompany a diagnosis of RCD.

In all patients data relative to the initial CD diagnosis, including serology and histology, should be carefully re-assessed. Evidence shows that in a variable percentage of cases a diagnosis of CD made in non-referral centers

turns out to be a wrong diagnosis at an accurate re-evaluation of serological and histological data and HLA genotyping [133, 137, 138].

Once the possibility of an initial misdiagnosis of CD has been ruled out, it is mandatory to make a thorough evaluation of compliance to the GFD as in patients with confirmed diagnosis of CD exposure to dietary gluten is the main cause of persistence of symptoms/signs [133, 137].

GFD compliance should be evaluated through a detailed dietary history and the use of food diaries; patients dietary habits should be investigated for inadvertent and/or voluntary gluten ingestion, taking into account potential causes of gluten exposure, such as frequent meals out and inadequate knowledge of gluten-containing foods. Gluten contamination and accidental ingestion of gluten are more common than perceived, and celiac patients tend to overestimate their adherence to the GFD [133, 137, 138].

In patients with evidence or suspect of gluten contamination dietary modification should be implemented as they result in symptoms resolution in the majority of patients at later time points during follow-up. It has been reported that episodic exposure to gluten is associated with a speedier onset and severity of symptoms than those observed in patients with chronic dietary gluten exposure [164]. These findings could reflect the greater immunological responses seen in the gut of patients reintroducing dietary gluten following a two-week GFD in comparison to chronic gluten exposure [165]. The partially/non adherent patients have not only greater probability to experience persistent symptoms but also enhanced risk to develop complications, including other immune-mediated diseases and malignancies [30, 166]. This reinforces the need for considerable physician counseling and extensive dietetic support in maintaining the GFD and identifying potential sources of gluten contamination.

If symptoms persist despite the confirmation of the initial diagnosis and the likelihood of adherence to the GFD, patients should be investigated for other causes that could explain symptoms/signs. Prevailing symptoms should drive in the choice of diagnostic investigations [133, 135-137].

In the first instance non-invasive tests, such as blood, stool tests and breath tests should be preferred. In case of negative results to first level exams, second level investigations should be pursued, including upper gastrointestinal tract endoscopy with duodenal biopsies, lower gastrointestinal tract endoscopy and radiologic tests which may vary from patient to patient depending on presenting symptoms [133, 135-137].

The initial investigations on NRCD patients should include serology, particularly EMA status and anti-DGP IgG antibodies, as a useful clinical tool

to separate patients with positive serology, more likely to be gluten contaminated, from those with negative serology, more likely requiring further investigations.

A careful medication history should also be obtained, with special attention to agents known to induce intestinal damage such as nonsteroidal anti-inflammatory drugs (NSAIDs), proton-pump inhibitors (PPI) or olmesartan [108-109, 167-169].

CD and IBS can coexist and failure to attain optimal subjective well-being can be seen in CD patients with concomitant IBS. In these patients, adequate compliance with a GFD is not sufficient to resolve symptoms or, in some circumstances, can paradoxically exacerbate some IBS-related symptoms, such as constipation, due to the low fiber intake.

A suggested algorithm to investigate non-responsive celiac disease patients is reported in **Figure 1**.

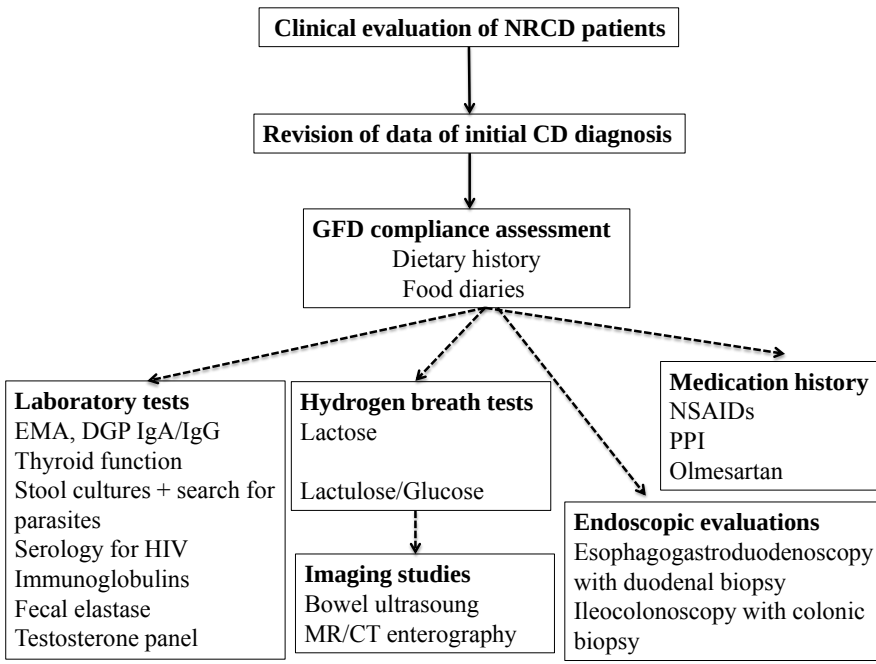

Figure 1. Suggested algorithm to investigate non responsive celiac disease patients *(Modified from Stasi* et al. *J Clin Gastroenterol. 2016 Mar;50(3):239-43).*

Non responsive celiac disease (NRCD); Celiac disease (CD); Gluten free diet (GFD); Anti-endomysial antibody (EMA); IgA/IgG anti-deamidated gliadin peptide

(DGP IgA/IgG); Human Immunodeficiency Virus (HIV); Magnetic resonance (MR); Computed Tomography (CT); non-steroidal anti-inflammatory drugs (NSAIDs); Proton Pump Inhibitors (PPI).

CONCLUSION

Non-responsive coeliac disease refers to a variety of conditions that can induce an unsatisfactory clinical response in coeliac patients. It often defines a temporary condition, which is subsequently clarified. It is of mandatory importance for the physician to discriminate between benign concomitant conditions and potentially serious complications of CD accounting for symptoms. NRCD occurs in nearly one fifth of celiac patients on gluten-free diet and its occurrence warrants further investigations in order to optimize the management of celiac patients.

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