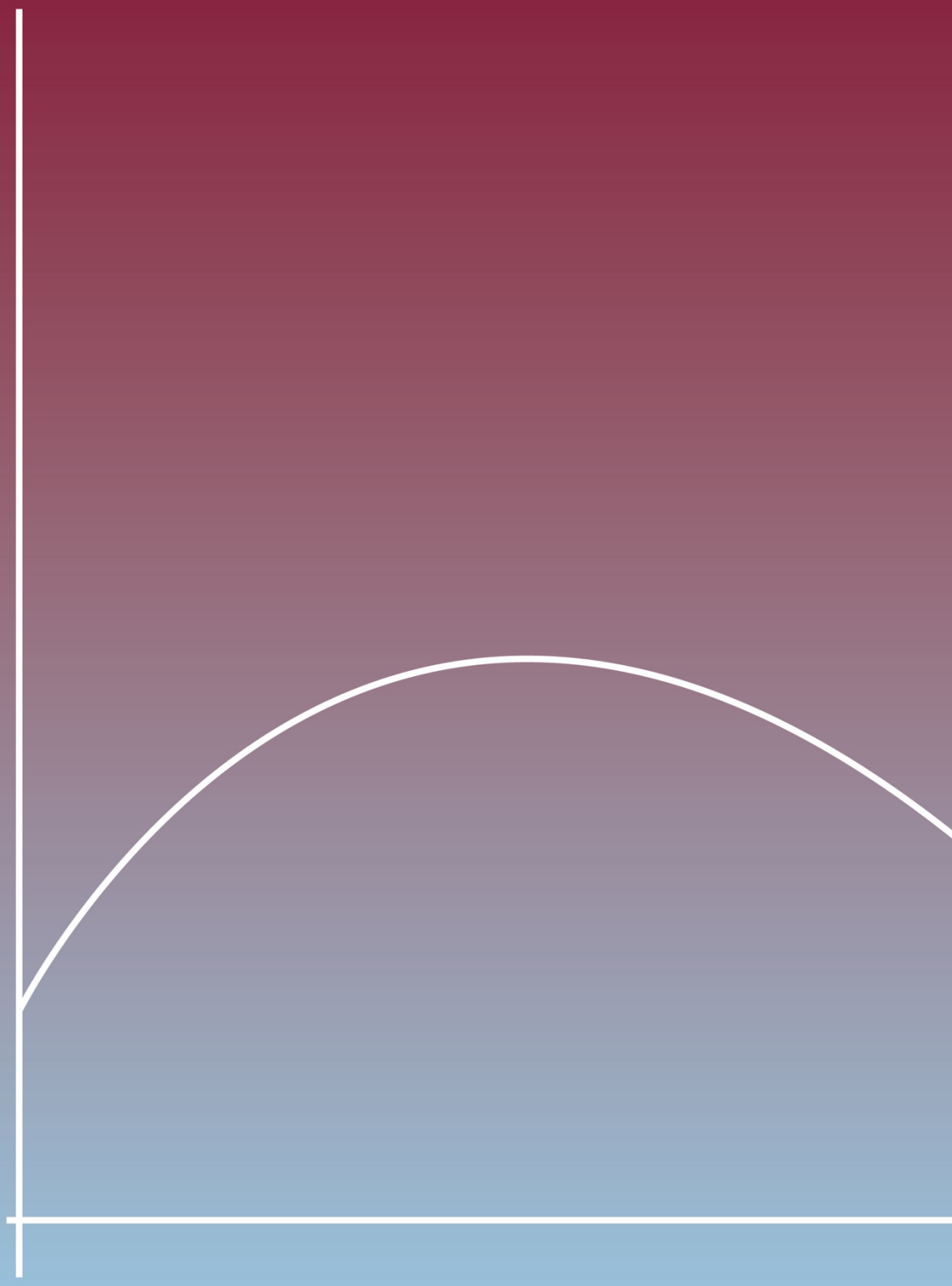

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Ph.D. course in
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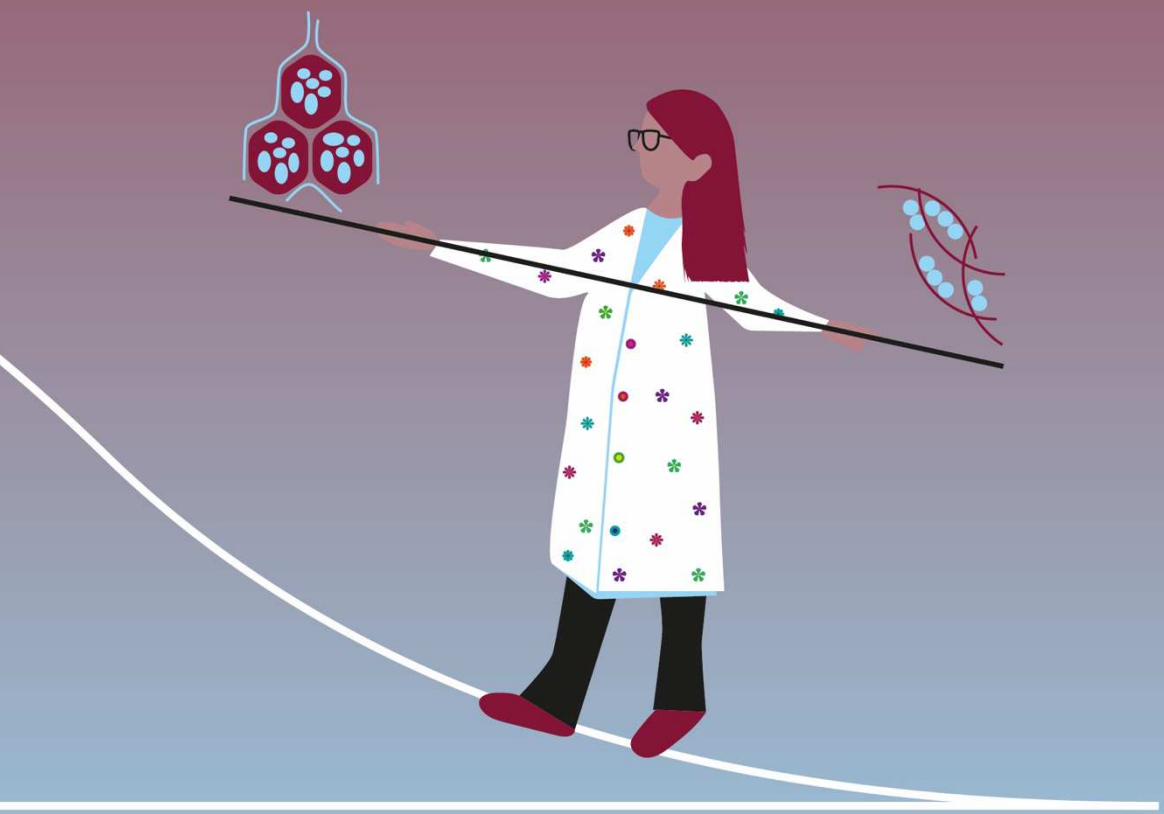
XXXVI cycle

Innovative approach to design cereal-based product with low glycemic response



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2024

“Per arrivare alla fonte bisogna
nuotare controcorrente”

Stanisław Jerzy Lec

A mio Padre che mi ha insegnato
a nuotare controcorrente

A mia madre e mia sorella che
mi hanno permesso di farlo

Table of contents

List of abbreviations

ANOVA	Analysis Of VAriance
AX	Arabinoxylan
BMI	Body Mass Index
BU	Brabender Units
CFW	Calcofluor White
CHD	Coronary Heart Disease
CHO	Carbohydrate
CI	Confidence Interval
CLSM	Confocal Laser Scanning Microscopy
CS	Coarse Semolina
CVD	Cardio Vascular Disease
ΔH	Transition enthalpy
DSC	Differential Ccanning Calorimetry
DW	Durum Wheat
G	Gluten
GIP	Glucose-dependent Insulinotropic Peptide
GLUT	Glucose Transporter
GR	Glycemic Response
IAUC	Increased Area Under the Curve
LD	Large Dough
LF	Large Flour
LPP	Large Pre-Proofed dough
MANOVA	Multivariate Analysis of VAriance
MD	Medium Dough
MF	Medium Flour
MPP	Medium Pre-Proofed dough
MT	Mixing Time
MW	Molecular Weight
NSP	Non-Starch Polysaccharide
PCA	Principal Component Analysis
PSD	Particle Size Distribution

RB	Rhodamine B
RDS	Rapid Digestible Starch
RH	Relative Humidity
Rmax	Resistance to extension
RR	Relative Risk
RS	Resistant Starch
S	Semolina
SD	Standard Deviation
SD	Small Dough
SDS	Slowly Digestible Starch
SEM	Sum Error of the Mean
SF	Small Flour
T2D	Type 2 Diabetes
To	Onset Temperature
Tp	Peak Temperature
TPA	Texture Profile Analysis
TS	Total Starch
WA	Water Absorption

Summary

Bread is a staple food consumed daily all over the world as the main source of energy. It is mainly produced with white wheat flour, it is palatable, affordable, and easy to incorporate into meals. However, white wheat bread is generally characterized by a high glycemic index due to the complete starch gelatinization and a porous structure that facilitates the enzymatic digestion of starch, leading to rapid glucose absorption. For these reasons, bread was reported as a main contributor to the glycemic index of the diet and in turn to its glycemic load. Following a low glycemic load diet, compared to a high glycemic load diet, has been proven to reduce the risk of major non-communicable diseases, including type 2 diabetes, cardiovascular disease, and colorectal cancer. Therefore, reducing the glycemic index of bread is considered a promising strategy for lowering the overall glycemic index of the diet. Until now, several approaches have been used, however, these strategies often have a detrimental effect on bread textural properties, reducing consumer acceptability. Limiting the accessibility of α-amylase to starch through physical barriers would be a promising alternative strategy to modulate starch hydrolysis and, at the same time, obtain bread with good textural features. This Ph.D. project aimed to investigate, from the micro- to macro-structure, the effect of physical barriers, such as intact cell walls, protein matrix, and food texture, and their interactions, on the starch digestibility of bread.

In the first two studies, described in Chapters 2 and 3, the effect of increasing flour particle size, and therefore increasing the presence of intact cells, on starch digestibility of durum wheat and rye bread was evaluated. From each cereal, flours with three particle sizes were produced, i.e., small (<350 μm), medium (1000 μm-1800 μm), and large (>1800 μm). For both cereals, the presence of clusters of intact cells

microscopically detected in medium and large flour decreased the starch digestibility in the flour, acting as a barrier between starch and digestive enzymes. However, this protective effect was lost after bread production. It was hypothesized that the long mixing time, needed to reach an optimally developed dough, increased the cell wall porosity due to the solubilization of their components, thereby enhancing enzyme penetration. Moreover, the use of coarse flour (>1000 μm) reduced the cohesiveness of breadcrumbs, increasing the disintegration rate during digestion and, in turn, the starch accessibility. Based on these results, in Chapter 4, the effect of reduced mixing time and the increase in breadcrumb cohesiveness on textural features and in vitro starch digestibility of bread made with coarse durum wheat flour (semolina, >1000 μm) was evaluated. Two approaches were tested to modulate bread cohesiveness: decreasing dough hydration and substituting 20% coarse semolina with vital gluten. Gluten-enriched bread samples, despite the use of different dough hydration and mixing times, exhibited improved textural properties (lower hardness and higher cohesiveness) and a slight decrease in starch digestibility at the end of in vitro digestion compared to bread made with 100% coarse semolina. This was attributed to the preservation of the structure during the digestion of bread thanks to gluten substitution, which could have limited crumb disintegration and consequently starch digestibility. In conclusion, substituting 20% coarse semolina with gluten was effective in enhancing the overall quality of coarse semolina bread and reduced in vitro starch digestibility. In Chapter 5, these findings were validated in a human study. Sixteen healthy volunteers participated in glycemic and insulinemic response tests, which were carried out following the ISO guidelines, and randomly consumed three bread samples. Bread samples were: bread made with 80% coarse semolina and 20% gluten (80CS_20G), its counterpart made with fine semolina (80FS_20G), and a sample made with fine semolina and a 5% gluten substitution (95FS_5G). The oral processing of bread samples was also evaluated to

assess the effect of gluten and semolina particle sizes on oral disintegration and the release of reducing sugars after mastication. No differences were found in glycemic response or mastication behavior among the three samples. However, the combined effect of gluten and coarse semolina (80CS_20G) resulted in a lower release of reducing sugars during the initial phase of starch hydrolysis, leading to a reduced insulinemic response after 30 minutes of bread consumption compared to the 80FS_20G sample. Additionally, confirming what is already reported in the literature, the compact structure of 95FS_5G elicited a lower insulinemic response compared to its more voluminous counterpart (i.e., 80FS_20G) thanks to a lower amount of reducing sugar release after mastication.

In conclusion, throughout the thesis, the effect of different physical barriers was evaluated from cereal flour to bread. Our results suggest that, in a processed matrix such as bread, only the combination of multiple factors, such as the use of coarse semolina (rich in clusters of intact cells) and gluten (able to increase the crumb cohesiveness) is a promising strategy to decrease bread starch digestibility, and in the same retain good textural properties.

General Introduction
Aim and scope

1.1 Durum wheat

Durum wheat (DW, Triticum turgidum L. ssp. durum (Desf.) Husn.), genome BBAA, is a tetraploid cereal grain produced and consumed all over the world since ancient times. Its production can be traced back to 6500-7500 years ago, however, it became an extended crop only 1,500–2,000 years ago (Maccaferri et al., 2019). Half of the global production is concentrated in the Mediterranean Area (Algeria, Turkey, Italy, Morocco, Syria, Tunisia, France, Spain, and Greece), furthermore, outside Europe, Canada, Mexico, U.S.A., Russia, Kazakhstan, Azerbaijan, and India also play substantial roles as producers of DW, with the first three being the most significant exporters (Martínez-Moreno et al., 2022).

Around seventy per cent of durum wheat kernel is composed of starch with amylose making up around 22-30% of the total starch content, and amylopectin 70-78%. The starch granules exhibit a biphasic distribution, consisting of large A-type granules with lenticular shapes and diameters ranging from 20 to 25 μm, and smaller B-granules, which are roughly spherical shape and with a diameter of 5-6 μm. The protein ranges between 8-15%, of which 10-15% is albumin-globulin and 85-90% is prolamins-glutelin. Among them, gluten, a non-soluble protein composed of monomeric and polymeric proteins (glutenin and gliadins), plays a pivotal role in determining the quality of the final product (Biesiekierski, 2017). The gluten properties are mainly due to specific combinations of alleles at the storage protein loci. In durum wheat, the specific glutenin alleles at the low molecular weight (MW) locus Glu-B3 and at the high MW weight locus Glu-B1 confer high elastic recovery and gluten firmness, which are characteristics associated with pasta quality (Mastrangelo & Cattivelli, 2021). The quantity of non-starch polysaccharides (NSP) in the kernel is around 2%, mainly comprised of arabinoxylans and β -glucans. The lipid content remains relatively stable at around 1.9%, with linolenic acid being the predominant fatty acid. Moreover, the grains are

characterized by a bright yellow color due to the carotenoid pigment that ranged from 6.2 ± 0.13 mg/kg and is comprised of lutein, zeaxanthin, and β-cryptoxanthin (Saini et al., 2023).

The durum wheat kernel is characterized by a very hard and glassy-textured structure. Generally, DW is milled to remove the bran and germ from the endosperm and coarsely ground it. Due to the hardness of the grain, a high force is needed to crack the kernel and a complex procedure of progressive grinding and sieving is used. To reduce energy and increase the friability of the endosperm, durum wheat is often tempered to reach 17–17.5% grain moisture (Saini et al., 2023; Samaan et al., 2006). DW is ground at different extents depending on the final items to be produced. Semolina, used for pasta and cous cous production, typically ranges in particle size from 550 to 150 μm. Its granulometry changes depending on the method used for pasta production and the desired characteristics of the final goods (Lafiandra et al., 2022; Sicignano et al., 2015). For bread production, the required particle size is generally lower than 180 μm. Therefore, DW is usually reground several times (Pasqualone et al., 2019) producing a great amount of damaged starch and in turn high water absorption (Fadda et al., 2010).

DW is extensively utilized in various cereal-based products, mainly for pasta, which, in its numerous forms, (i.e.; spaghetti, macaroni, lasagna sheet and fresh pasta) represents the most widely manufactured and industrialized end-product of DW. However, principally in the Mediterranean region, it is also used in a variety of staple foods such as durum wheat bread, couscous, bulgur, and different types of flatbreads and sweets (Martínez-Moreno et al., 2022; Sissons, 2022). For what concern durum wheat bread, its popularity is increasing all over the world due to its peculiar sensorial and textural properties (Sanfilippo et al., 2023). Notably, this bread is characterized by a harder, yellowish crust and a more compact crumb when compared to bread produced with common wheat (Triticum aestivum) (Bianca et al., 2023; Sissons, 2008). These characteristics, coupled with the grain's

high water-holding capacity, contribute to prolonged shelf life and the preservation of its sensory qualities during extended storage (Rinaldi et al., 2015). The reground semolina, used for bread production, is characterized by a high amount of damaged starch which, during the dough kneading, competes with the gluten to link water. This leads to a weak final protein network which, in turn, results in bread with a compact structure and reduced volume, typical of traditional durum wheat bread (Fadda et al., 2010).

1.2 Rye

Rye (*Secale cereale* L.) belonging to the grass family *Gramineae*, is one of the major crops for bread production, second only to common wheat (Arendt & Zannini, 2013; Deleu et al., 2020). Despite being considered a minor cereal, rye contributes to 1% of the total world cereal production (Arendt & Zannini, 2013). Rye exhibits remarkable resistance to cold temperatures, making it suitable for cultivation in regions with harsh climates like Germany, Poland, Russia, Denmark, and Belarus, which collectively account for 85% of its global production (Arendt & Zannini, 2013; Kaur et al., 2021; Németh & Tömösközi, 2021). The composition of rye kernel predominantly comprises starch, ranging from 55 to 65%, with amylose accounting for 22-26% of the total. Starch consists of both large (A-type, 60-90%) and small (B-type, 10-40%) granules, with diameters of 23-40 μm and less than 10 μm, respectively. Rye protein content varies widely (8-15%), depending mainly on growth conditions. Albumins-globulins are the main proteins, accounting for 38–51% of total protein content, while prolamin and glutelin, which are storage proteins also known as secalins, represent 17–19% and 9–15% of the total protein content, respectively. Among cereals, rye has the highest dietary fiber content, ranging from 15 to 25%. The most prominent components of dietary fiber are arabinoxylans (AX) (7.5-11.5%), followed by β-glucans (1.5-3%), lignin (3%) and cellulose (2.6%) (Andersson et al., 2009; Arendt & Zannini, 2013; Dziki, 2022). Rye grain is also rich in various phytochemicals such as phenolic acids, phytosterols and several other bioactive compounds such as flavonoids and anthocyanins. The lipid content in rye is similar to that in wheat (2-3%), with linoleic acid being the major component (Németh & Tömösközi, 2021).

The milling process of rye, traditionally done using a roller mill, is very similar to the wheat milling process with some differences due to the distinct grain structure of rye. The tempering process is shorter compared to that for wheat because the soft rye endosperm requires

less time to raise the moisture content of the kernels up to 15% (Arendt & Zannini, 2013). The extraction rate for rye flour tends to be lower than that of wheat due to difficulties in separating the endosperm from the seed coat, especially in grains with a high content of non-starch polysaccharides. Additionally, rye flour requires a larger sifting surface than wheat due to its sticky nature. Throughout the rye milling process, various flour fractions and mill streams are produced, exhibiting variations in composition due to the irregular distribution of chemical components in the kernel. Therefore, rye flours are generally classified based on ash content, with the number of classifications varying from nation to nation (Aprodu & Banu, 2017; Arendt & Zannini, 2013; Németh & Tömösközi, 2021).

Nowadays, rye is used in the production of various traditional foods, including bread, pumpernickel, porridge flakes, and biscuits but also alcoholic beverage, such as rye beer and whiskies. It is also added to traditional wheat-based bakery products to reduce starch digestibility and subsequent glucose absorption (Deleu et al., 2020). Regarding rye bread, its distinctive structure is primarily attributed to its low gluten content, compared to wheat, and its high fiber content. The rye bread structure is mainly characterized by a continuous phase of starch granules embedded in a dense fiber matrix, predominantly composed of arabinoxylans (Juntunen et al., 2003). To achieve a good structural network, acidification is essential. For this reason, rye bread is traditionally prepared using sourdough, a natural mixture of lactic acid bacteria and yeasts (other than *Saccharomyces cerevisiae*), obtained by combining rye flour and water (Deleu et al., 2020). The resulting acidic conditions positively affect the swelling of rye AX, the breakdown of cell walls, and the solubilization of pentosans, fundamental for crumb formation. Despite the peculiar texture of rye bread, compact, dense, slightly sour, and humid, it is widely consumed and commercialized thanks to its several nutritional benefits. Consuming rye products has been shown to lead to a lower postprandial insulin response, prolonged glucose profile, and

increased satiety compared with wheat bread, and this effect was widely observed in several randomized controlled trials (Deleu et al., 2020; Jonsson et al., 2018). Several factors contribute to the beneficial effects of rye products consumption. Soluble dietary fibers, present in both the rye bran and endosperm, increase digesta viscosity, slowing gastric emptying and reducing carbohydrate digestion and absorption in the intestines (Rosén et al., 2009). Additionally, bioactive compounds like phenolic acids may impede carbohydrate hydrolysis, stimulate insulin secretion, and inhibit enzymatic activity (Jonsson et al., 2018; Rosén et al., 2011). Jenkins et al. (Jenkins et al., 1986) also demonstrated the key role of the firm structure of rye bread (i.e., pumpernickel) in slowing down the glycemic response limiting the physical disintegration of the product during the digestion.

1.3 Starch structure

Starch is the most abundant carbohydrate found in nature and represents the major source of energy for both man and animals. It is mainly found in seeds, roots, and tubers, but also in stems, leaves, fruits and even pollen (Bertoft, 2017). Amylose and amylopectin, two polysaccharides, make up almost all the starch granules. Amylose is a linear molecule of α -(1–4)-linked D-glucose units and, depending on the botanical origin, represents 15-35% of the starch granules. Amylopectin, which ranges between 72 and 82% of the total starch, is a highly branched molecule, with a-(1–4)- linked D-glucose backbones and about 5% of α-(1–6)-linked branches (Buléon et al., 1998; Magallanes-Cruz et al., 2017; Pérez & Bertoft, 2010). Amylose and amylopectin are arranged in granules resembling rings or shells known also as "growth rings", as shown in Figure 1.1. These rings exhibit alternating amorphous and semi-crystalline structures, typically measuring in total 100-400 nm in thickness. The semi-crystalline portions, approximately 9 nm within starch granules, arise from the linear and short external chain segments of amylopectin with a degree of polymerization of approximately 10–20 glucosyl units. The structure of the amorphous rings, instead, is poorly known, but it is generally believed that consists mainly of amylose (Bertoft, 2017; Vamadevan & Bertoft, 2015). The granules display distinct morphologies in different plants, ranging from circular, oval, and ogival to elongated, flat, lenticular, or polyhedral and their diameters span from 1 μm to over 100 μm upon their botanical origin (Pérez & Bertoft, 2010; Vamadevan & Bertoft, 2015). Notably, potato starch granules have the largest granule, ranging from 2.7 to 70.7 μm, while rice starch granules are the smallest, measuring less than 10 μm (Guo et al., 2023). In cereals, as already mentioned for rye and DW starch, the starch presents a bimodal size distribution of granules, consisting of A-type starch with a disk-like or lenticular shape (10–35 μm) and B-type starch, roughly spherical or polygonal in shape ranging from 1

to10 μm (Peng et al., 1999). A- and B-type starch granules are reported to have significantly different functional properties and chemical composition in terms of amylose, amylopectin, lipid, and protein content (Guo et al., 2023; Peng et al., 1999). It has been hypothesized that the different morphology, size and shape of starch granules play a significant role in determining the physical, chemical, and functional attributes of starch. Smaller starch granules can absorb more water per unit area, and they are more prone to aggregation than the big ones. Larger starch granules exhibit notable swelling and are susceptible to breakage during the process of paste formation. Other factors, such as the ratio between amylose and amylopectin could influence physical and functional characteristics of starch (Vamadevan & Bertoft, 2015).

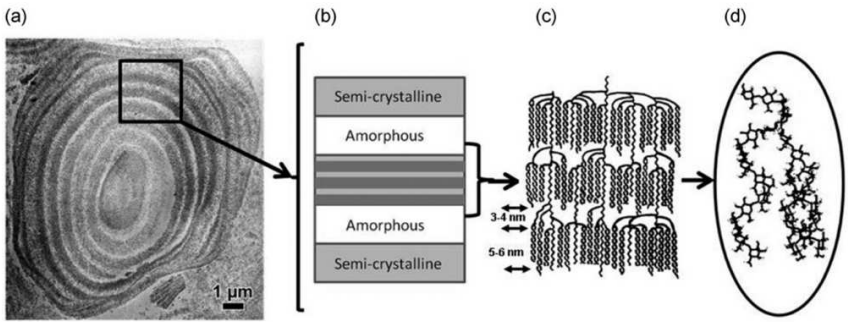

Figure 1.1. From growth rings to amylopectin. Schematic representation of the several levels ofultrastructure of starch: (a) Ultrathin section of maize starch; (b) alternation of semi-crystalline and amorphous rings; (c) clustered model of amylopectin; (d) branching a double helix onto a single helix (Pérez & Bertoft, 2010).

1.4 Starch digestion and glucose absorption

The digestion of starch starts in the mouth where the food is chewed and mixed with saliva, which contains α-amylase. Alpha amylase, including pancreatic amylase, is an endosaccharidase that hydrolyzes internal α-1,4 glycosidic bonds, but it does not attack the branch point with α-1,4- or α-1,6- glycosidic bonds. The optimal pH for the enzymatic activity of α-amylase is 7 but the enzyme is stable for a large range of pH values (5.0 to 10.5) (Goodman, 2010; Sky-Peck, 1977). Therefore, salivary α-amylase remains active until the food bolus is fully disintegrated and the pH does not drop down to 5 in the stomach. Consequently, up to 30–40% of complex carbohydrates can be digested before the food reaches the small intestine (Goodman, 2010). When chyme enters the duodenum, it stimulates the release of pancreatic enzymes, which are then delivered into the lumen via the hepatopancreatic sphincter (sphincter of Oddi). Moreover, the low-pH chyme emptied from the stomach stimulates the release of secretin, which, in turn, stimulates the exocrine pancreas to release bicarbonate to neutralize the pH of the chyme and optimizes the environment for the activity of the intestinal enzyme. Pancreatic amylase enters the intestinal lumen and actively breaks down complex carbohydrates into maltose, maltotriose (also known as isomaltose), trisaccharides, oligosaccharides, and α-limit dextrins (oligosaccharides with branching points) (Berdanier & Berdanier, 2021). The generated oligosaccharides (i.e., maltose, maltotriose, and α-dextrins) undergo further degradation through the action of disaccharidases. These enzymes are situated as membrane-integrated proteins on the brush borders of intestinal epithelial cells, known as enterocytes. These brush-border membrane enzymes exhibit different specificities and are positioned at various locations within the small intestine. They function as exoenzymes, cleaving individual monosaccharides from

oligosaccharides or transforming disaccharides into monosaccharides. These enzymes, such as β-glucoamylase and isomaltase, are involved in the complete hydrolyzation of starch. β-glucoamylase, also known as maltase, specifically hydrolyzes 1,4 glycosidic linkages between glucose molecules in maltose or the residue at the end of the oligosaccharide tail. Isomaltase, also known as limit dextrinase or debranching enzyme, hydrolyzes 1,6 glycosidic bonds at branch points in limit dextrins and 1,4 linkages in maltose and maltotriose. The brush border enzymes are distributed in the small intestine, with limited activity in the duodenum and distal ileum, and no activity in the large intestine. Isomaltase has a high distribution and activity in the proximal jejunum, whereas glucoamylase has its highest activity in the proximal ileum (Goodman, 2010).

The resulting monosaccharides are absorbed by enterocytes through specific transport proteins. Active transport mechanisms, specifically the sodium-dependent carrier-mediated transporter SGLT1, facilitate the absorption of glucose and galactose. This transporter takes advantage of the Na^+ gradient (i.e., low intracellular Na^+ concentration), which is created by basolateral Na^+, K^+-ATPases, to bring hexoses into the enterocytes (Goodman, 2010). Subsequently, D-glucose can leave the cell on its basolateral side via facilitated diffusion transporters, i.e., sodium-independent transporter, GLUT2. It diffuses the exoses from a high concentration inside the cell to a low concentration outside the cell. GLUT2 is a member of GLUT family of glucose transporters, each transporter is unique for specific tissue and facilitates the transfer of glucose through the cellular membrane in the body (Goodman, 2010). Carbohydrates, whose bonds are not attacked by amylolytic enzymes, moved to the lower part of the intestine, as not digestible starch, where they are hydrolyzed and fermented by intestinal microbiota.

1.5 Glucose regulation and homeostasis

The pancreas through the opposing action of glucagon and insulin effectively regulates and maintains stable glucose levels in postabsorptive state. This stability is reached through a balance between the production and utilization of glucose (Röder et al., 2016). The mechanism is known as glucose homeostasis, and it is briefly summarized in Figure 1.2. After a meal, the rate of glucose absorption depends on the meal carbohydrate content and the rate of glucose absorption. This can lead to exogenous glucose entering the bloodstream at more than double the rate of endogenous glucose production. The elevation in blood glucose leads to a decline in glucagon level, while insulin, the primary glucoregulatory hormone, is released from the pancreatic β-cells (Giugliano et al., 2008). Insulin, primarily suppressed the endogenous glucose production and accelerated glucose utilization by liver, muscle, and adipose tissue. Additionally, it drives the storage of glucose in the liver as glycogen, this process is called glycogen synthesis, but also leads to the incorporation of amino acids into proteins and lipogenesis (Giugliano et al., 2008). Once glycogen stores are filled, glucose surplus from the diet is converted and stored as triacylglycerols in the adipose fat depots and, in times of need, this fat can be oxide. Then, during periods of fasting such as sleep or between meals, when blood glucose level decreases, the pancreas releases from α-cells glucagon. Glucagon is a potent hyperglycemic hormone, which stimulates hepatic glycogenolysis and glucose synthesis, leading to the release of glucose in the bloodstream, to be utilized mainly by the brain. In this way, the glucose returns to its steady state (Berdanier & Berdanier, 2021; Röder et al., 2016).

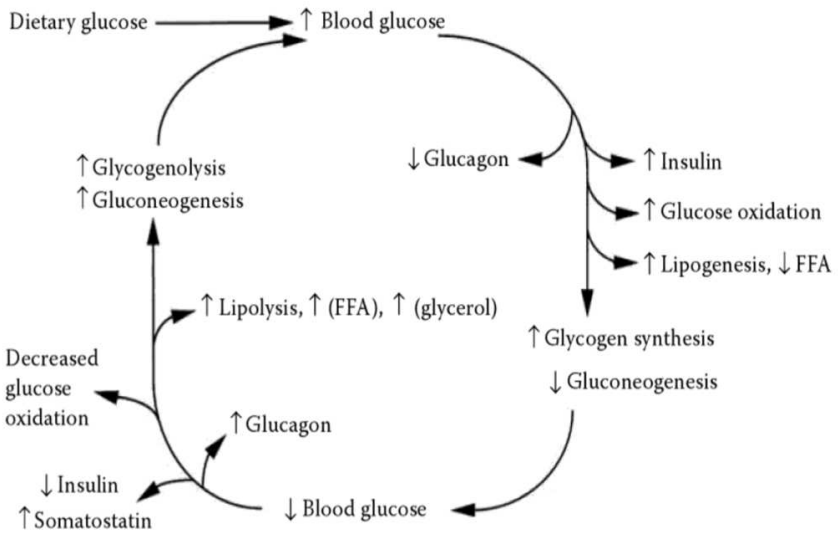

Figure 1.2. Schematic overview of glucose homeostasis. When the blood glucose level rises due to the dietary glucose intake, blood insulin level rises and glucagon level falls. Glucose oxidation increases, as does glycogenesis and lipogenesis. Gluconeogenesis falls. As the blood glucose level falls, this process reverses. Glucagon level rises, insulin falls; glycogenolysis and gluconeogenesis rise, and peripheral glucose oxidation decreases (Berdanier & Berdanier, 2021).

1.6 Postprandial hyperglycemia

In healthy people, the average fasting blood glucose concentration (no meal within the last 3 to 4 hours) is between 80 to 90 mg/dL [4.4 -5 mmol/L], and, after a meal, the postprandial glucose ranges between 120 to 140 mg/dL [6.6 -7.8 mmol/L], but the body's feedback let the glucose returns to basal level within 2 hours (Giugliano et al., 2008). Hyperglycemia, instead, occurs when fasting blood glucose surpasses 110 mg/dL [6.1 mmol/L] or when postprandial glucose levels are higher than 140 mg/dL [7.8 mmol/L] two hours after eating (Gerich, 2003). The term "hyperglycemia" is derived from the Greek hyper (high) + glykys (sweet/sugar) + haima (blood) and it is used to describe a state in which blood glucose concentration is elevated beyond the normal range. As

described in the previous paragraph, the body usually maintains glucose homeostasis through a delicate balance between the increase in blood glucose during the fasting state through the action of glucagon and the subsequent glucose-lowering effects driven by insulin. The mechanisms behind the development of hyperglycemia are still not certain, one theory proposes that food overconsumption, particularly diets abundant in easily available carbohydrates, can overwhelm adipose tissue's capacity to handle the excess energy and this can disrupt insulin signaling (Meza et al., 2019). This leads to insulin resistance, defined as a condition where a known amount of endogenous insulin cannot trigger the absorption or the use of glucose as much as it would in a normal population. The glucose in excess remains in the blood circulation in response to inadequate energy uptake (Kosmas et al., 2023). The sharp increases in blood glucose levels consequent to insulin resistance induce oxidative stress, that, in combination with soluble advanced glycation end products and lipid peroxidation products, acts as key activators of upstream kinases. These processes contribute to endothelial dysfunction and the expression of inflammatory genes (Node & Inoue, 2009). Moreover, the damage to the endothelium is characterized by phenotypic changes, inflammation, altered permeability and reduced endothelium-dependent dilation. These alterations in endothelial functions often anticipate many of the issues observed in type 2 diabetes (T2D) and cardiovascular disease (CVD) (Chen et al., 2019; Meza et al., 2019). Moderate energy restriction, regular physical activity, and dietary behavior changes have proven to be effective in regulating postprandial hyperglycemia. Among promoting dietary factors, as previously mentioned, postprandial hyperglycemic response seems to be mainly influenced by the overconsumption of carbohydrates. However, not only the quantity but also the type of carbohydrates and other macronutrients consumed in parallel are determinants in modulating postprandial glucose concentration and responses (Papakonstantinou et al., 2022; Vlachos et al., 2020).

1.7 Glycemic index, glycemic load, glycemic and insulinemic response of food

As already mentioned, the composition of the diet, especially the quality and the quantity of consumed carbohydrate-rich foods, plays a central role in the control of glycaemia. In 1981, Jenkins et al. (1981) developed the concept of glycemic index (GI), an index used to classify different sources of carbohydrate (CHO) and CHO-rich foods according to their effect on postprandial glycaemia (Brouns et al., 2005). GI is commonly described as the incremental area under the curve of blood glucose response (IAUC), caused by the consumption of a given test food containing 50 g of available carbohydrate expressed as a percentage of the response elicited by 50 g reference food (either glucose solution or white bread) in the same subject (Wolever, 2013). The glycemic index ranges from 0 to 100, and, generally, foods are classified in: low- (GI <55), medium- (GI 56–69) or high-GI (GI>70) (Choi et al., 2012), as shown in Figure 1.3. GI is an index representing the quality of carbohydrates and is measured by a standardized method defined by ISO (ISO-26642, 2010). However, most of the time, starchy foods are consumed in different amounts and portions, and, since GI does not consider the amount of carbohydrate ingested itself, another index was developed to calculate the combined effects of quantity and quality of carbohydrates, i.e., the glucose load (GL) (Carneiro & Leloup, 2020). GL describes the effects of quantity and quality of carbohydrate in a food and is calculated as the product of GI and the carbohydrate amount (in grams) of the food item divided by 100 (Chiu & Taylor, 2011). GI and GL are indices of the product itself, representing the quantity and quality of carbohydrate foods. However, the calculated GI of a mixed meal is not necessarily expected to predict its glycemic response (GR), defined as the postprandial blood glucose response (change in glucose concentration) elicited

when a composite food or meal containing carbohydrates is ingested (Augustin et al., 2015). This is because the impact of a mixed meal on glycemia is influenced not only by the quality and quantity of carbohydrates but also by factors such as the rate of gastric emptying and the presence of various food ingredients, including fats and proteins.

Jenkins et al. (2006) demonstrated that the addition of almonds to meals decreased the GR of the rice. Moreover, it was proven that the co-ingestion of oil, chicken breast and vegetables with white rice significantly diminished the GR of white rice (Sun et al., 2014). Similar results, i.e., a decrease in GR, were found when the mashed potatoes, a high GI product, were ingested in a complete meal, with the addition of oil, chicken breast and salad (Hätönen et al., 2011). Not only do other foods ingested along the starchy food modulate its GR, but also the composition of the preceding meal can impact the body GR. This phenomenon is known as the "second meal effect". As reported by several authors, the consumption of a breakfast high in protein or high in fiber (barley, rye kernel, or product rich in amylose), has the potential to influence the GR to white-wheat bread during the subsequent meal (Brighenti et al., 2006; Iversen et al., 2022; Meng et al., 2017; Nilsson et al., 2008). This effect is believed to be driven by products of colonic fermentation, including organic acids and notably short-chain fatty acids, which are produced together with gas during carbohydrate fermentation in the colon (Brighenti et al., 2006).

The insulin index is another indicator developed to characterize the metabolic effect of food. This index measures the incremental insulin response over 120 minutes following the consumption of approximately 1000 kJ (239 kcal) of a test food, relative to the response after consuming a 1000-kJ (239 kcal) portion of a reference food (analogous to the GI, using either glucose or white bread) (Behbahani et al., 2023; Holt et al., 1997). Unlike GI or GL, the insulin index offers additional insights because insulin secretion is not only triggered by carbohydrate intake (Vlachos et al., 2020). Meals containing meat,

despite having minimal carbohydrates, can still elicit an insulin response. Moreover, foods rich in protein or the incorporation of protein into a carbohydrate-rich meal can prompt a moderate increase in insulin secretion without raising blood glucose levels. Similarly, the addition of a substantial amount of fat to a carbohydrate-rich meal elevates insulin secretion even though plasma glucose responses are reduced. Consequently, postprandial insulin release does not always correspond proportionally to blood glucose levels or the total carbohydrate content of a meal (Holt et al., 1997).

Overall, all these different indices are useful for characterizing meals and diets in terms of the quality and quantity of carbohydrates. Moreover, despite the above-mentioned limitations, GI and GL have been widely used in epidemiologic studies for the characterization of diets, due to their ease of calculation and strong correlation with estimated postprandial glycemia in healthy individuals (Bao et al., 2011).

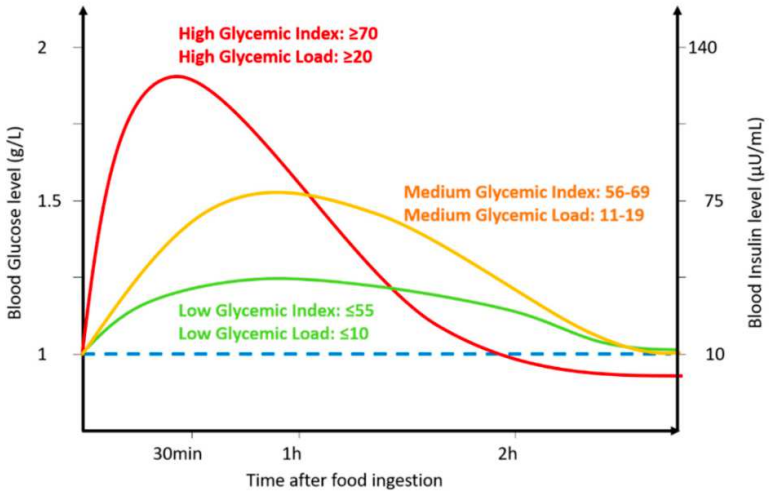

Figure 1.3. Schematic chart depicting the impact of meals with varying glycemic index (GI) or glycemic load (GL) levels on blood glucose (shown on the left axis) and insulin (displayed on the right axis). Low vs. medium vs. high GI or GL and their corresponding value range are indicated (Carneiro & Leloup, 2020).

1.8 High GI *vs* low GL diets and the risk of non-communicable diseases

Epidemiological data suggest that low GI and GL diets play a crucial role in reducing the risk of non-communicable diseases such as CVD, coronary heart disease (CHD), stroke, cancer, and T2D. Describing the huge quantity of these studies is out of the scope of this thesis. However, recent meta-analyses measuring the relation between the risk of major non-communicable diseases and the quality of the diet in terms of GI and GL as well as the main hypothesized mechanisms are briefly described.The relation between CVD and a high GI or GL diet was investigated through a meta-analysis involving 15 prospective cohort studies (9 in Europe, 4 in the USA, 1 in Japan, and 1 in Australia), encompassing 438,073 individuals, with follow-ups ranging from 5 to 25 years. For measuring dietary habits, 12 studies utilized validated food-frequency questionnaires (FFQs), while the remaining 3 studies employed diet records or diet history interviews. All primary studies were adjusted for age, body mass index (BMI), smoking, physical activity, alcohol consumption, cereal fiber, and total energy intake. Higher dietary GI levels were associated with a significant 13% increased risk for CHD (relative risk (RR) 1.13 95% confidence interval (CI) 1.04–1.22). Additionally, higher dietary GL levels were associated with a significant 28% increased risk for CHD (RR 1.28, 95% CI 1.14–1.42). However, when stratifying by gender, high GL and GI diets were significantly associated with an increased risk of CHD in women but not in men (Fan et al., 2012). In the same meta-analysis, an association between categories of GI and GL and stroke risk was observed, analyzing a total of 3 studies comprising 130,739 participants and 1,894 incident stroke cases. The results suggested that only high dietary GL, not high GI, was associated with a 19% increased risk of stroke (RR = 1.19; 95% CI 1.00–1.43) (Fan et al., 2012). The mechanism

behind these associations was explained by the positive effects of reducing GI/GL in the diet on glycemic control, such as fasting blood glucose, hemoglobin A1C, insulin sensitivity, and fasting insulin in the healthy population. Furthermore, significant benefits in BMI or weight loss, total cholesterol, and low-density lipoprotein were observed after the consumption of a low GI diet. Reducing GL intake also showed improvements in both systolic and diastolic blood pressures, while low GI diets affected only diastolic blood pressure (Dwivedi et al., 2022). A systematic review, which included 75 epidemiological reports covering over 147,090 cancer cases, evaluated the relationship between a high GI or GL diet and the risk of cancer (Turati et al., 2015). This meta-analysis comprised case-control studies (32) and prospective studies (43) carried out in 20 different cohorts all over the world (23 from Europe, 20 from America, 6 from Asia, and 3 from Australia). Nearly all studies utilized validated FFQs to assess dietary habits. Among the studies, there were variations in the adjustment for potential confounding factors, the majority (93%) adjusted for BMI/physical activity and energy intake, 70% for indicators of social classes, and 65% for tobacco consumption. While all RRs were above unity, no significant associations were found, except for colorectal cancer risk, which had a 16% increased risk with a high GI (RR=1.16, 95% CI: 1.07–1.25) (Turati et al., 2015). This meta-analysis was updated in 2019 (Turati et al., 2019), analyzing a total of 88 studies. This update largely confirmed the results of the previous meta-analysis and provided further results for bladder cancer (GI: 1.25, 95% CI: 1.11–1.41—GL: 1.10, 95% CI: 0.85–1.42, 4 studies) and kidney cancers (GI: 1.16, 95% CI: 1.02–1.32—GL: 1.14, 95% CI: 0.81–1.60, 5 studies) (Turati et al., 2019). Epidemiologic studies have found associations between elevated risk of cancers and increased insulin levels, which could promote carcinogenesis either directly by stimulating the production of insulin receptors or indirectly by suppressing insulin-like growth factor binding proteins (Renehan et al., 2004). However, the weak association between GI and GL and the

risk of cancer may be attributed to the fact that postprandial insulin concentrations do not change proportionally with the blood GR (Holt et al., 1997). Consequently, GI or GL may not reliably predict insulin secretions, and therefore, their association with cancer risk remains uncertain (Choi et al., 2012). The relation between T2D and a high GI or GL diet was assessed through a meta-analysis involving 10 studies that exclusively utilized valid dietary tools (Livesey et al., 2019). The average number of participants in each study ranged from 2,000 to 40,000, with three studies comprising 60,000 to 70,000 participants. The meta-analysis encompassed data from various countries and regions, including the USA, Japan, Europe, China, and Australia. The majority of the studies assessed dietary exposures using FFQs, with one study employing a diet history questionnaire and another utilizing an undefined questionnaire in conjunction with a structured interview conducted by a trained dietician. All studies were adjusted for major potential confounders. The combined T2D–GI RR was 1.27 (1.15–1.40) per 10 units GI, while that for the T2D–GL RR was 1.26 (1.15–1.37) per 80 g/d GL in a 2000 kcal (8400 kJ) diet (Livesey et al., 2019). In summary, diets characterized by elevated GI or GL were strongly linked to the occurrence of T2D in the general population. Several physiological mechanisms have been proposed to elucidate the positive association of GI and GL with T2D. Diets with high GI and GL are known to stimulate insulin production, leading to a state of hyperinsulinemia, which, in turn, can induce insulin resistance. Additionally, the chronic exposure to elevated concentrations of blood glucose and free fatty acids induced by high-GI and -GL diets may contribute to β-cell failure, disrupting insulin signaling (Bhupathiraju et al., 2014).

1.9 The relative contribution of each food group to overall dietary glycemic load

Several epidemiological studies have been carried out across culturally diverse countries and among individuals with different lifestyles, education, gender, and age to assess the primary contributors to GI and GL in dietary patterns all over the world. These epidemiological investigations covered several regions in Europe (Rodríguez-Rejón et al., 2014; Van Bakel et al., 2009), and Australia (Louie et al., 2017). The findings from these studies consistently revealed that, despite the social status, gender, or geographical location, white bread and bread rolls consistently came out as the predominant contributors to dietary GL. Fruits ranked second in influencing dietary GL, with a more pronounced impact in Mediterranean regions (Rodríguez-Rejón et al., 2014) and among female populations (Van Bakel et al., 2009). In contrast, regional variations were more pronounced for other carbohydrate-rich foods. Potatoes play a significant role in dietary GL among populations from Northern European countries (including the Netherlands, Austria, Sweden, and Denmark), as well as Australia (Louie et al., 2017; Rodríguez-Rejón et al., 2014; Van Bakel et al., 2009). Conversely, pasta emerged as a substantial contributor, particularly in Italy. Regarding legumes, their contribution to overall dietary GL and GI was relatively minor, accounting for only 1.2% of GL and 1.3% of GI. Notably, among both alcoholic and non-alcoholic beverages, discernible north-south gradients were observed, with higher contributions observed in northern regions compared to southern ones. Beer was the primary contributor to dietary GL among alcoholic beverages, with a significant impact observed primarily in men living in northern European centres (Van Bakel et al., 2009). As briefly discussed in the previous paragraph, a low GI and GL diet can be beneficial in reducing the risk factors of mainly CHD and T2D.

Therefore, reducing the GI of bread, the main contributor to the GL in the diet, might be fundamental in decreasing the overall GI and GL of the diet.

1.10 Strategies to reduce the glycemic index in bakery products:

The case study of wheat bread

Bread is a staple food consumed all over the world as the main source of energy. It is a simple product that is basically made by mixing a few ingredients: wheat flour, yeast, water, and salt. The flour and water are kneaded in a dough with yeast, the dough is allowed to rise and then shaped into various forms and sizes. The daily consumption is driven by factors such as affordability, palatability, and ease of incorporation into meals or snacks. In developed countries, the average consumption of bread per capita, generally produced with wheat flour, less often with rye and other cereals, stands at approximately 70 kg per year (De Boni et al., 2019). Bread is generally characterized by a high GI (Atkinson et al., 2008; Scazzina et al., 2016) due to the complete starch gelatinization and a porous structure that facilitates enzyme-starch interaction, leading to rapid hydrolysis and subsequent glucose absorption. For these reasons, bread was reported as a main contributor to GL in dietary patterns across multiple countries (Louie et al., 2017; Rodríguez-Rejón et al., 2014; Van Bakel et al., 2009). Therefore, there is a need to reduce its starch digestibility, and in turn its GI, with a specific emphasis on that produced with wheat flour. Until now, several strategies have been explored to address this issue. Increasing the soluble fiber content by adding bran, whole grains, fruit fiber, legume-based flour, seeds or carob (Behall et al., 1999; Eelderink et al., 2017; Kurek et al., 2018; Mandalari et al., 2018; Stamataki et al.,

2017) is one of the strategies largely applied for lowering GI of wheat bread. The addition of fiber increases digesta viscosity, which slows down the gastric emptying and reduces carbohydrate absorption in the small intestine acting as a barrier between starch and α-amylase (Rosén et al., 2009). Moreover, using flour rich in amylose reduces starch swelling and gelatinization compared to regular wheat flour. This contributes to the retention of starch crystalline structure during baking, delaying enzymatic hydrolysis thanks to reduced enzyme diffusion within the granules (Arp et al., 2018; Hoebler et al., 1999; Li et al., 2022). Furthermore, incorporating polyphenols or other bioactive compounds in bread recipes can inhibit the activity of primary digestive enzymes, i.e., α-amylase and α-glucosidase, by binding their catalytic sites. Polymeric polyphenols can also precipitate with digestive enzymes, forming non-digestible complexes (Kan et al., 2020, 2022). Moreover, the fermentation method can significantly affect GI of bread. Sourdough fermentation and subsequent dough acidification can increase resistant starch and enhance the release of peptides, free amino acids, polyphenols, and water-soluble dietary fiber. These compounds can bind to the catalytic sites of enzymes, limiting starch digestibility, promoting fast gastric emptying and stimulating satiety hormones (Katina et al., 2007; Nionelli et al., 2018; Rizzello et al., 2016). However, most of these techniques have a detrimental effect on the texture, e.g., volume, taste and color of bread, limiting the consumer acceptability (Khalid et al., 2017; Navrotskyi et al., 2019). Starch digestibility depends, amongst others, on the contact between α-amylase and the starch (Englyst et al., 1996; Heaton et al., 1988; Shevkani et al., 2017). Therefore, the preservation of the native microstructure (cell wall integrity) and employing processing techniques to create a macrostructure (protein network and food matrix) can be used to decrease the accessibility of starch, ensuring acceptable textural properties and lowering glycemic response. For the aim of the thesis, it was chosen to investigate the effect of the natural physical barrier starting from the microscopic

level: the effect of cell wall integrity and the presence of protein network; to the macroscopic level, the food matrix and consequentially the disintegration during the oral processing and intestinal digestion. In the subsequent sections, these strategies are analyzed and discussed in detail.

1.10.1 Effect of cell wall integrity

Starch granules are encapsulated within plant cells, which are arranged side by side to form the plant tissue. Each cell is constituted by an outer, semi-rigid, and hydrophilic cell wall and an inner lipophilic cell membrane. The external part of the cell wall is formed by a mixture of cellulose, hemicellulose and pectin (in varying proportions depending upon the botanical origins) and provides structural support and physical barriers (Dhital et al., 2016). Preserving the integrity of the cell wall has become a foremost topic in recent decades in the fields of food technology and nutrition, as a promising strategy to modulate starch and protein digestibility (Dhital et al., 2016). It was demonstrated that isolated intact legume cells, including chickpeas, peas, mung beans, and red kidney beans, can resist the acidic condition of the stomach and the enzymatic activity and peristaltic movements of the small intestine, preserving their structure, and, as a result, limiting the starch and protein hydrolysis. Conversely, broken cells, without a physical barrier, become vulnerable to digestive enzymes, leading to easy nutrient digestion. The mechanism behind this restricted enzyme digestion is a multifaceted interplay of factors. Intact cells within the plant structure can act as barriers reducing the interaction between starch and enzymes. Additionally, the compact and thick cellular structure could control the diffusion of enzymes inside the cells retarding their diffusion. Furthermore, cell wall materials' strength, thickness, and composition can influence water diffusion, thereby restricting starch gelatinization. Moreover, the non-catalytic binding of digestive enzymes to cell wall components could also limit and retard the rate

and extent of starch hydrolysis (Bhattarai et al., 2017; Dhital et al., 2016; Rovalino-Córdova et al., 2018, 2019). Besides the use of isolated legume cells, several studies were also conducted in pulse flours, rich in clusters of intact cells, as ingredients in complex food products, such as bread. *In vivo* experiments demonstrated that replacing 30% or 60% (w/w) of wheat flour with a patent chickpea intact cell powder in bread led to approximately a 40% reduction *in vivo* glycemic response compared to white bread rolls (containing around 50 g of available carbohydrates and 12 g of wheat protein per serving). Microscopic analysis confirmed the presence of intact cell walls even after the baking process, highlighting the crucial role of cell integrity in moderating glycemic responses in these products (Bajka et al., 2021). However, the processing could significantly impact the integrity of cell walls and consequently affect their ability to restrict starch accessibility. As shown by Pallares Pallares et al. (2018), despite the thick and tightly structured cell walls of legumes, prolonged cooking times could increase starch digestibility due to the enhancement of the cell walls porosity. For what concerns cereals, the main ingredient of bakery products, the effect of cell wall integrity in decreasing starch digestibility is ambivalent. In isolate cell walls of wheat and sorghum, the starch digestibility is significantly lower in intact cells than in the damaged ones, confirming the ability of cell walls to act as a barrier between starch and enzyme and to limit starch gelatinization (Bhattarai et al., 2018; Korompokis et al., 2019). Furthermore, Edwards et al. (2015a) showed a significant decrease in blood glucose concentration (33%) after consumption of coarse porridge, rich in clusters of intact cells, in ileostomy volunteers compared to the glucose concentration elicited by the consumption of smooth porridge. This confirms the impact of cell wall encapsulation on limiting starch digestibility in minimally processed food matrices. However, when coarse flour containing intact cells was used to create more processed foods like bread, this effect seemed to be lost. Korompokis et al. (2021) found the rate of *in vitro* starch digestibility

of bread made with the inclusion of coarse flour did not significantly differ from that in control bread, even though microscopic images showed that the integrity of the cell walls was maintained after the baking process. It is noteworthy to mention that bread has a much more complex matrix than isolated cells and undergoes several hydrothermal processes. It was hypothesized that during the mixing and fermentation steps, the porosity of the cell walls increased, thereby enhancing the accessibility of amylase to starch granules. These mixing and fermentation steps could be responsible for the activity of endogenous enzymes on arabinoxylans and β-glucans in wheat cell walls (Dornez et al., 2008). Such activity could have increased the solubilization and structural redistribution of these components and the consequent increase of cell wall porosity. The different digestibility behavior between intact cells of wheat and chickpeas in bread matrix can be attributed to the intrinsic differences in cell tissue properties, especially the permeability of cell walls to amylase diffusion. Chickpea cotyledon cells, for instance, have thick walls (~1–2 μm) able to restrict starch amylolysis even after thermal processing (Bajka et al., 2021). Wheat endosperm cells are instead visibly thin, and the shape is less defined (~0.6–1.0 μm) (Edwards et al., 2021). Additionally, another potential mechanism that could explain the lost effect of intact cell walls in reducing starch digestibility in bread made with coarse flour is the weakening of the gluten network due to the incorporation of large particles (Bressiani et al., 2017). It has been demonstrated that a dense compact gluten network can entrap starch granules and limit their accessibility to amylolytic enzymes. By contrast, the incorporation of large particles inside the dough could increase the fracturability of breadcrumbs, increasing the disintegration rate of bread during digestion, and, consequentially, the accessibility to starch. However, the mechanism responsible for the diminished protective effect of intact cells in bread remains uncertain. It is unclear at which stage of bread processing the cell wall loses its ability to function as a barrier, limiting the contact between

starch and digestive enzymes. Furthermore, to the best of our knowledge, research in this field has predominantly focused on wheat which is characterized by a thin and fragile cell wall among cereal grains. Other cereals, such as rye, which is characterized by a uniform and thicker cell wall compared to wheat grain, have not been extensively studied yet in this context (Autio & Salmenkallio-Marttila, 2001).

1.10.2 Effect of endogenous and exogenous protein

Besides starch, protein is the second macronutrient present in cereals, and it also has a central role in modulating starch digestibility both when it is added as ingredients in food preparation, but also as an endogenous component inside the cereal (Lu et al., 2022). In simple model systems, adding different protein hydrolysates (rice, wheat, soy, whey) to native starch (wheat, potato, rice, maize) induces a considerable decrease in its *in vitro* digestibility (Bhattarai et al., 2016; Chen et al., 2019; Lu et al., 2021). The addition of protein significantly increases the strength of hydrogen bonds and, enhancing starch molecule order, limits the starch swelling, the degree of gelatinization, and consequentially the rate of starch hydrolysis. Furthermore, the hydrolysate can inhibit the amylolytic enzyme binding the sites available for the enzyme, thereby inhibiting the digestion of starch (López-Barón et al., 2017). Therefore, the mechanism behind the reduced starch digestion in the presence of proteins involves a combination of hydrophobic interactions and hydrogen bonding between hydrolysates/peptides and starch, along with the inhibition of amylolytic enzyme activity (Liu et al., 2021; Lu et al., 2023; Xiong et al., 2023). Moreover, wheat gluten protein, a major component of wheat grain, and gluten peptides naturally formed during digestion, not only inhibit α-amylase more effectively than isolated soy protein but also act as a barrier, limiting starch accessibility, when the gluten network

is formed (Chen et al., 2019; Xiong et al., 2023). Gliadin and glutenin, which are the main storage proteins of wheat, accounting for 85-90% of the total amount of protein. After hydration and mixing force, it is formed a discontinuous network named gluten that surrounded the starch granules (Li, Li et al., 2021). This dense compact structure, naturally formed in several bakery products, such as bread and pasta, could decrease starch accessibility, limiting the contact between starch and enzyme (Chen et al., 2019). Xu et al. (2021) demonstrated that by increasing the density of gluten/ gluten hydrolysates the rate and extent of starch digestion decreased in a food model made with maize starch and gluten. This effect is confirmed in pasta, where the lower glucose release during digestion compared to bread, and, in turn, its low GI, must be researched in its dense compact structure given by the strong gluten network that surrounds the starch granules (Scazzina et al., 2016). Besides physically hampering the contact between starch and enzyme, the protein network also limits the starch swelling and gelatinization, maintaining intact the crystal structure of the starch granule (Zou et al., 2016). Vanhatalo et al. (2022) showed that the pasta consumption in healthy volunteers leads to lower acute postprandial glycemic and insulinemic responses when compared with bread, prepared with the same ingredients, but with a more porous easier-to-disintegrate structure. Moreover, inside the pasta digesta, light microscopy images showed that starch remained partially unchanged, due to the encapsulation in the protein network, whereas the protein network of bread largely disappeared due to extensive hydrolyzation during gastric digestion. These results indicate that pasta has a strong protein network that is slowly digested and protects starch granules from enzymatic activity (Vanhatalo et al., 2022). To confirm the effect of endogenous gluten network in modulating starch digestibility, it is interesting to notice that gluten-free products elicit higher glycemic responses than their normal gluten-containing products (Berti et al., 2004), underlying the role of protein in decreasing starch digestibility.

1.10.3 Effect of food matrix

The food texture, which is formed during food processing, represents a macrostructure able to modulate the starch digestibility of starchy products. Comparing starchy foods like bread, breakfast cereals, and pasta, it is evident how the processing influences their structure and, consequently, starch digestibility (Mishra & Monro, 2012). Leavened bread and puffed cereals, such as puffed rice and cornflakes, exhibit the highest starch digestibility among starchy foods due to their porous aerated architecture resulting from extensive disruption of the cereal through starch gelatinization and thermal processes (Mishra & Monro, 2012). In these products, the starch is completely gelatinized and swelled, and the macrostructure makes a poor resistance to disintegration under digestion conditions leading to easy access by the amylolytic enzymes (Štěrbová et al., 2016) and resulting in products with high GI (Atkinson et al., 2008). By contrast, in pasta or rolled oats, the starch is encapsulated in a matrix less digestible, and this results in food products with medium or low GI (Atkinson et al., 2008). Pasta, with its homogeneous consistent macrostructure, maintains high strength during digestion, restricting enzyme diffusion and preserving food particle integrity (Vanhatalo et al., 2022). During the oral and gastric phases, bread particles disintegrate into small and shreds particles, while pasta particles remain larger and smooth-rimmed. This difference in surface area influences enzymatic susceptibility, resulting in bread being more susceptible due to its larger surface area compared to the compact structure of pasta, which is more resistant to enzymatic access (Vanhatalo et al., 2022). It is also known that, within the same group of products such as bread, the different crumb densities and structures can effectively influence its starch digestibility.

Jenkins et al. (1986), firstly, point out the role of the structure in lowering the starch digestibility of pumpernickel, a traditional rye bread, compared with common wheat bread. The dense compact

structure of pumpernickel limits its disintegration during digestion, leading to bigger particles of digesta, more resistant to enzymatic diffusivity, compared to the ones of common bread. Several researchers exploited this hypothesis, comparing the starch digestibility of bread with the same composition and different densities, and found bread with the hardest and most dense structure, the least digestible compared to soft voluminous bread (Freitas et al., 2022). Steam processing and hydration levels further influence bread texture and digestibility, leading to products with denser structures and reduced disintegration rate compared to traditional bread (De La Hera et al., 2014; Lau et al., 2015; Martinez et al., 2018). Together with the modification of structure, the hydration depletion in the dough might also lower the starch gelatinization in the crust, increasing the amount of slowly digestible starch. A reduced volume and high density, behind its role in decreasing the disintegration rate of bread, could also increase the satiety index, demonstrating that the modification of bread structure can also favorably alter metabolic and appetite responses (Burton & Lightowler, 2006). Bread texture can modulate digestibility mostly when it changes the disintegration rate during digestion. However, it is fundamental to consider that the first step in the disintegration pathway during digestion is mastication. Bread texture has a central role also in modulating oral processing and this could contribute to individual differences in glycemic response to foods, especially in plant tissue where chewing behavior can modulate the release of starch from the cellular matrix (Gao et al., 2015; Gao & Zhou, 2021; Pentikäinen et al., 2014). Different chewing times could lead to bolus particles differing in size and shape: the smaller the particles, the faster the starch hydrolysis, the bigger the particles, the longer the disintegration rate during the gastric-intestinal digestion (Nordlund et al., 2016). Moreover, the saliva impregnation and the mechanism of bolus hydration could also influence digestibility. Salivary α-amylase is the first enzyme in the pathway of starch digestion and its activity depends, mainly, on the quantity of saliva

inside the bolus (Jourdren et al., 2016). Bread with different textures, moisture content and density leads to different rate of saliva production and thus, its modulation, can significantly alter the glycemic response (Pentikäinen et al., 2014).

Understanding how these mechanisms affect starch digestibility and hydrolysis will enable the modulation of bread glycemic response by manipulating its structural features.

Aim and thesis outline

In recent decades, the consumption of energy-dense diets, primarily composed of highly digestible starchy foods like bread, along with a global increase in obesity rates and a sedentary lifestyle, has emerged as a major contributor to the development of non-communicable diseases such as cardiovascular diseases and type 2 diabetes. Therefore, reducing the glycemic response of bread is a worthy strategy to modulate the overall glycemic impact in the diet. Until now, several approaches have been used. However, these strategies can have a detrimental effect in the bread textural properties limiting consumer acceptability and, consequently, its consumption.

This Ph.D. project aims to investigate, from micro to macro levels, the effect of physical barriers, such as cell walls, protein matrix, and food texture, and their interactions, on the starch digestibility of bread products. The final aim is to develop bread products with acceptable textural properties while having a lower glycemic response compared to common bread.

To achieve this objective, four experimental projects were conducted, as shown in Figure 1.4.

The first study, detailed in **Chapter 2**, aimed to monitor the integrity of durum wheat cell walls and their ability to limit starch digestibility throughout all stages of the baking process, from raw flour to dough and bread. Semolina was produced with varying particle sizes: small (<350 µm), medium (1000 µm-1800 µm), and large (>1800 µm). The presence of intact cell walls was examined using confocal laser scanning microscopy, and starch digestibility was assessed using the *in vitro* starch digestibility method (Englyst's method) at different stages of bread production. The ultimate goal was to determine at which stage the effectiveness of the physical encapsulation of starch within cell walls in reducing *in vitro* starch digestibility is lost.

The objective of the second study, outlined in **Chapter 3**, was to assess the effectiveness of clusters of intact cells on the starch digestibility

of rye flour and a rye bread model. To assess that, three flours varying in particle size were obtained: small (<350 µm), medium (1000 µm-1800 µm), and large (>1800 µm). The presence of intact cell walls was verified using confocal laser scanning microscopy in both rye flour and bread models. The textural quality, *in vitro* starch digestion (Englyst's method), and physical disintegration during the digestion were investigated to deeply study the relationship between the integrity of cell walls, structural features of bread, and *in vitro* starch digestibility.
In **Chapter 4**, the effects of dough mixing time, gluten addition, and dough hydration on the bread texture and starch digestibility of durum wheat bread made with coarse semolina (>1000 µm) were explored. Six durum wheat bread samples were prepared using either coarse semolina alone (>1000 µm) or with 20% vital gluten substituting semolina, two hydration levels, with 70% water (optimal water absorption) and 55% (low water absorption), and different mixing times (3.5 or 45 min). Different mixing times and water absorption were tested to assess the effect on the textural properties and starch digestibility (Englyst's method) of bread samples. The ultimate aim of this project was to produce a bread with coarse semolina exhibiting acceptable textural properties and low *in vitro* starch digestibility for subsequent *in vivo* testing.
The aim of the fourth project, detailed in **Chapter 5**, was to investigate the effects of coarse flour and 20% gluten addition in durum wheat bread on the postprandial glycemic and insulinemic responses in healthy volunteers. This was done by comparing the metabolic responses of such bread to those of a standard durum wheat bread made with fine semolina, as well as those of a durum wheat bread made with fine semolina and 20% gluten. Furthermore, the oral processing of bread samples was investigated to understand how gluten and particle sizes of semolina influence oral disintegration, the release of reducing sugars after mastication, and subsequently, the glucose and insulin responses.

Finally, **Chapter 6** summarizes and integrates the main findings of all the chapters presented in this thesis. Furthermore, the scientific challenges and future perspectives were also highlighted.

Ph.D. Journey

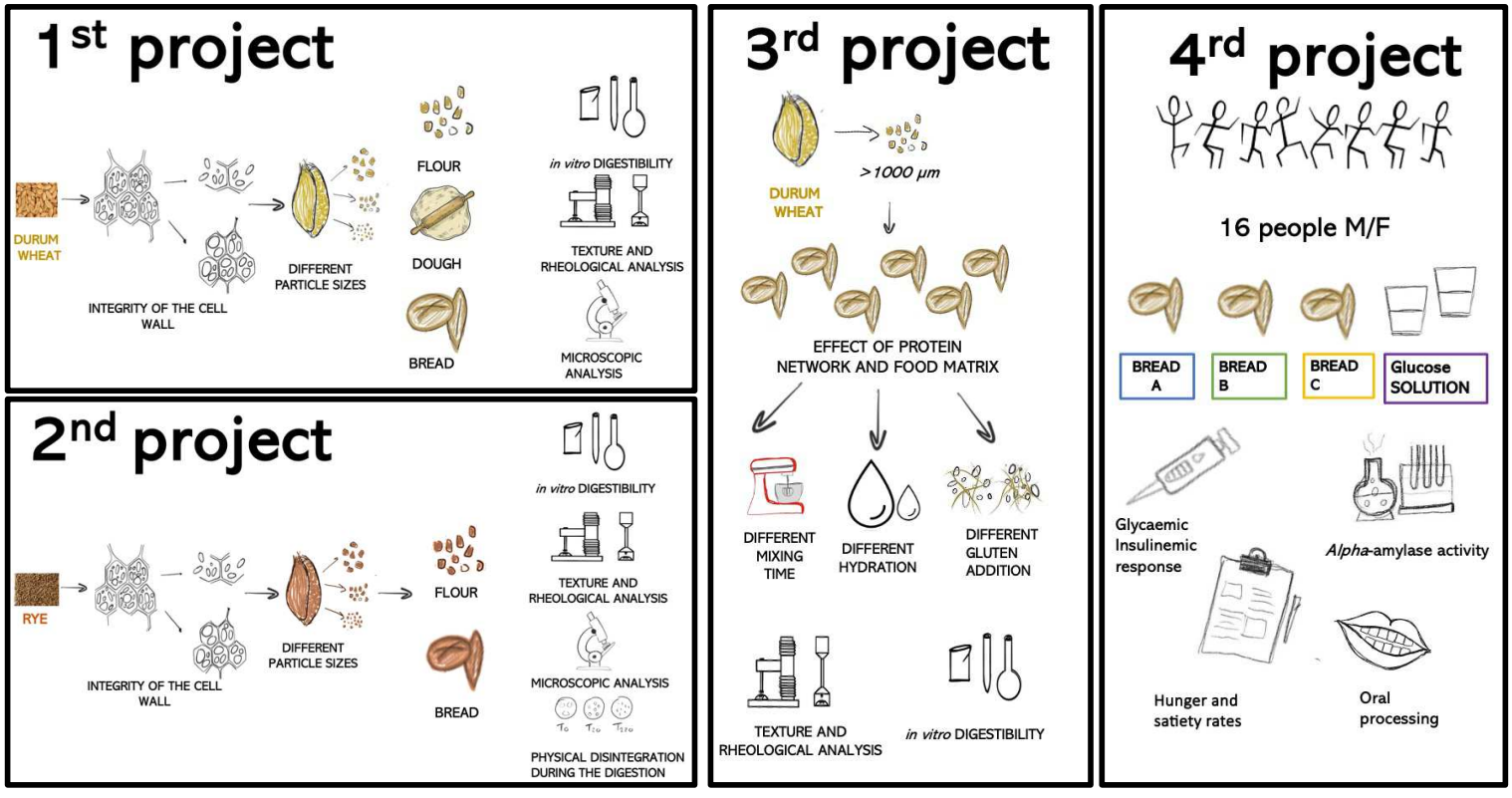

Figure 1.4. Schematic overview of the Ph.D. journey

Monitoring the effect of cell wall integrity in modulating the starch digestibility of durum wheat during different steps of bread making

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Abstract

Reduction of starch digestibility in starchy foods is beneficial for lowering the risks for major non-communicable diseases. Preserving cell integrity is known to delay starch digestibility in flour but its effect in bread is not clear. In this study, the effect of increasing particle size on *in vitro* starch digestibility of durum wheat flour, dough, and bread was investigated. Cell integrity was retained during bread processing for medium (1000 μm-1800 μm), and large (> 1800 μm) flour, whereas in small one cell walls were mostly damaged (< 350 μm). *In vitro* starch digestibility of flour decreased increasing particle size, but no difference was found in dough. In bread, instead, a modest decrease of starch digestibility for the bread made by large particle was observed, likely due to its dense structure. In conclusion, a high particle size could limit starch digestibility in durum wheat flour but not in bread.

Keywords:

Wheat durum; particle size; intact cells; *in vitro* starch digestibility; bread quality; confocal laser scanning microscopy.

2.1 Introduction

Durum wheat (*Triticum turgidum* L. subsp. *durum* Desf.) bread, mainly consumed in Southern Europe, is increasing its popularity due to the characteristic flavor, taste and prolonged shelf life (Giannone et al., 2018). Durum wheat bread, as most bread types, is characterized by a highly porous structure and fully gelatinized starch. It has a high glycemic index (GI) due to the rapid digestion of starch in the human digestive tract (Fardet et al., 2006). It has been demonstrated that consuming high GI foods is a risk factor for the development of chronic diseases, including type 2 diabetes and cardiovascular diseases (Blaak et al., 2012). Most strategies aiming at lowering the GI of wheat bread have focused on increasing the fiber content through the addition of bran, whole grains, fruit fiber, legume-based flour, seeds, or carob (Andrzej et al., 2018; Fardet et al., 2006; Scazzina et al., 2013). Next to this, the use of resistant starch or amylose, the addition of polyphenols, and different bread-making technologies have been investigated (Kan et al., 2020; Stamataki et al., 2017). Most of these strategies have a detrimental effect on the bread features. The addition of fiber, bran and coarse grains was demonstrated to have a negative effect on the gluten index, farinogragh stability and loaf volume (Khalid et al., 2017; Navrotskyi et al., 2019). Moreover, the inclusion of aleurone could affect the flavor of bread, resulting in less acceptance due to the intense odor and bitter taste (Bagdi et al., 2016). Therefore, researchers are looking for strategies able to reduce the bread digestibility without strongly compromising its textural and sensory properties.

Digestion of starch is carried out by salivary and pancreatic α-amylase (Englyst et al., 1996; Pellegrini et al., 2020). In intact plant tissues, amylase access to starch is limited by the presence of a cell wall made up of indigestible polysaccharides (Rovalino-Córdova et al., 2018). Generally, to produce flour for bread making purpose, the cereal grains are milled to obtain a particle size around 180 μm. This causes

a great disruption of the cell walls in which the starch granules are entrapped, thus increasing the contact between starch and amylase. It has been hypothesized that increasing flour particle size may be a suitable strategy to lower starch digestibility by limiting α-amylase accessibility to starch (Korompokis et al., 2019). Larger particles have a lower surface-to-volume ratio and a higher percentage of well-preserved intact cells than smaller particles. This decreases the amount of starch that escapes from the cell and is easily digested by amylases (Englyst et al., 1992). Several studies have investigated the starch digestibility of wheat endosperm products differing in particle size. (Mandalari et al., 2018) demonstrated that *in vitro* starch digestibility was inversely proportional to wheat particle size. (Edwards et al., 2015a) confirmed this effect in ileostomy patients where the glucose, which was released after intestinal digestion of porridge meals made with coarse and fine particle sizes, decreased as the particle size of the flour increased. This last study demonstrated that wheat endosperm cell walls retain their integrity after 4 h of digestion. However, contradictory results were found when the effectiveness of flour particle size was investigated in bread. (Lin et al., 2020) found that increasing particle size of whole wheat flour significantly decreased starch digestibility in bread, whereas (Korompokis et al., 2021) demonstrated that the incorporation of coarse flour did not have an effect in modulating the rate of starch digestibility in bread. These results suggest that during bread processing the efficacy of flour particle size in modulating starch digestibility is lost.

Therefore, in the present study, the effect of flour particle size on starch digestibility is studied in raw flour, dough and bread, to better understand how the processing affects the ability of cereal cell wall to act as a barrier to enzyme accessibility. The ultimate aim is to determine at which stage the physical encapsulation of starch within cell walls can effectively reduce the starch *in vitro* digestibility.

2.2 Materials and methods

2.2.1 Materials

Peeled durum wheat grain was purchased from Duru BakliyatTM (Hediklik Diş Buğdayı, Turkey). Rhodamine B R6626 (≥95%, Sigma Aldrich), and Calcofluor White M2R (Fluorescence Bright 28, MP Biomedicals) were used as dyeing agents for confocal laser scanning microscopy. The enzymes for *in vitro* digestion procedure (all from Sigma Aldrich) were: (1) pepsin P7000 (from porcine gastric mucosa, specific activity ≥ 250 units/mg solid); (2) pancreatin P7545 (from porcine pancreas, 8 x USP); (3) invertase I4504 (from baker yeast, specific activity ≥ 300 units/mg solid); and (4) amyloglucosidase A7095 (from *Aspergillus niger*, ≥ 260 U/mL). All other chemicals and solvents used were of analytical grade.

2.2.2 Methods

2.2.2.1 Flour preparation

To obtain three particle sizes, durum wheat was milled with a pin mill (Multi-mill, Alpine Hosokawa, Augsburg, Germany). The parameters of the milling were as follows: rotational speed: 1500 rpm (25 Hz), power: 1000 W, difference pressure filter: 30.0 bar. The milled wheat flour was sieved through three sieves: 1800 µm,1000 µm and 350 µm (Gilson company, Lewis center, USA). The fraction above 1800 µm was classified, and from now on indicated as, large flour (LF), and the fraction smaller than 1800 µm but larger than 1000 µm was classified as the medium fraction (MF). A part of the medium fraction was further milled (rotational speed: 8000 rpm (133.3 Hz)) to obtain the small fraction < 350 µm (SF).

2.2.2.2 Dough preparation

The dough was prepared according to a standard recipe with 1.2% yeast and 1% salt as % wet flour basis (Table 2.1) (Tagliasco et al., 2021). To obtain dough with the same consistency (500 Brabender Units), the amount of water and the mixing time was optimized. These values were determined for each dough performing a water absorption test at 30 °C and 63 rpm (1.05 Hz) with a Farinograph (Brabender GmbH & Co KG, Duisburg, Germany). All the ingredients were mixed at room temperature (20 ±1 °C) with a Hobart mixer (N50, Hobart, Woerden, The Netherlands). The dough was divided into a loaf of 90 g, manually moulded and placed in a small pun bread (7.7 x 4.7 x 4.0 cm) (Amsterdam, The Netherlands). The fermentation was conducted in a proofing cabinet at 30 °C and 75% relative humidity (RH). This proofing cabinet was prepared by adding an excess saturated solution of NaCl in an enclosed borosilicate desiccator glass DSGL300 (United Scientific Supplies, USA) and then inserting the desiccator in an incubator to maintain the temperature stable. The proofing time for each bread sample was set to unify the cumulative gas production during the leavening. The baking tins containing the dough were placed in a proofing cabinet type SDCC-1P/W (Koma koeltechnische Industrie B.V, Roermond, NE) at 30 °C with a pressure of 760 bar. The fermentation time of SF was set at 90 minutes. The carbon dioxide (CO_2) production was measured during the fermentation of the dough with the Risograph (National Manufacturing C., Lincoln, NE) and the data were collected by the software Risosmart (National Manufacturing C., Lincoln, NE). To establish the proofing time of medium flour and large flour dough, the fermentation time for each dough was set to produce the same amount of CO_2 as SF dough produced in 90 minutes of fermentation. In the following paragraph, the terms “dough” would be used to indicate the sample after the proofing step. Moreover, small dough (SD) is referred to the dough made with small particle size (<350 µm), medium dough (MD) prepared with medium particle size (1000 µm-1800 µm) and large dough (LD)

obtained with large particle size (>1800 µm). For the starch digestibility analysis, the dough was also analyzed before the proofing step. In this case, the pre-proofed doughs obtained by the different particle size flours were named respectively small pre-proofed dough (SPP), medium pre-proofed dough (MPP) and large pre-proofed dough (LPP). The dough preparation was repeated three times.

Table 2.1. Formulation of durum wheat bread made with small (< 350 µm) medium (>1000 µm; <1800 µm) and large (>1800 µm) particle size. The ingredients are expressed on 100 g of flour.

	Small	**Medium**	**Large**
Flour (**g**)	100	100	100
Water (**g**)	71	71.5	73
Salt (**g**)	1	1	1
Yeast (**g**)	1.2	1.2	1.2
Mixing time (**min**)	5	60	90
Proofing (**h**)	1.30	1.45	2.00

2.2.2.3 Bread preparation

The proofed dough was baked at 200 °C for 20 minutes in a hot air oven (OV425CS; 1,800 W, 230 V, 50 Hz, Inventum, Arnhem, The Netherlands). After baking, the bread was taken out of the aluminum pun bread (7.7 x 4.7 x 4.0 cm) cooled for 60 minutes on an oven metallic rack (Inventum, Arnhem, The Netherlands) at room temperature (20 ± 1 °C), and stored in Ziploc® (SC Johnson, USA) quart freezer plastic bag 17.7 x 18.8 cm. Two loaves were immediately placed at -20 °C for starch digestibility analysis and CLSM experiments. Three loaves were kept at room temperature (20 ± 1 °) and analyzed in terms of quality characteristics, the day after the production. The bread-making was repeated three times.

2.2.2.4 Flour characterization

The moisture content of the flours was measured following the official method (Method AACC 44-15.02., 1999). Total starch content was determined using the assay kit supplied by Megazyme (Bray, Ireland) following the procedure: Determination of total starch content of samples containing resistant starch (RTS-NaOH Procedure - Recommended). Damage starch was quantified according to AACC Method 76-31.01 with the assay kit supplied by Megazyme (Bray, Ireland). The protein content of flour samples was conducted following the Dumas method using a flash EA 1112 NC analyzer (Thermo Fisher Scientific Inc., Waltman, USA) following the manufacturer protocol. The conversion factor for wheat protein was 5.83 (Müller, 2017). All the analyses underwent triplicate measurements.

2.2.2.5 Bread characterization

The moisture content of bread was measured following the official method (Method AACC 44-15.02., 1999). Water activity was determined using an a_w-meter (Labmaster-aw, Novasin, Lachen, Switzerland) at 25 °C. Specific loaf volume was determined with the rapeseed displacement method according to the official method (Method AACC 10-05.01, 2009). The rapeseed was purchased form Beduco NV (Schoten, Belgium). The specific volume was calculated as the ratio between the volume and weight (cm^3/g). The measurement was done twice for each bread production. A texture profile analysis (TPA) using a TA-XT plus analyzer (Stable Micro Systems, Godalming, UK) was performed to measure the textural properties of bread. Bread was cut into slices of 2 cm with a kitchen bread knife and shaped in a cylinder of 2 cm in diameter. Bread was squeezed twice with a 75.0 mm diameter cylinder probe P/75 and a load cell of 50 kg. The applied probe test speed was 1.00 mm/s, trigger force of 0.049 N, 40% strain and rest time of 5 seconds. The force (N) was calculated over time (min)

for each sample. The parameters studied from the TPA were hardness (N): the maximum force achieved at the first bite and springiness (-): the degree to which a deformed material returns to its original state after the force is removed. Each bread was sliced into three parts and analyzed.

2.2.2.6 Starch digestibility

Starch digestibility was determined according to the colorimetric determination described by (Englyst et al., 1992). Precisely 2 grams of flour, pre-proofed and proofed dough, and bread of the three particle sizes were analyzed. The flour and the doughs were weighted as they were, the bread crumb was portioned in small cubic pieces (*ca.* 5 x 5 x 5 mm). The methodology consisted of two phases: the gastric (30 minutes) and the intestinal (120 mininutes). In the gastric phase, the sample, after the addition of 10 mL of pepsin (≥ 250 units/mg solid)–guar solution (0.05 M HCl), was shaken at 180 rpm (3 Hz) in a water bath for 30 minutes at 37 °C. For the intestinal phase, 5 mL of enzyme mixture, made by pancreatin (8 x USP), invertase (≥ 300 units/mg solid) and amyloglucosidase (≥ 260 U/mL), was added to the gastric digesta together with 10 mL of 0.25 M sodium acetate buffer (37 °C) and 5 marbles. The samples were incubated at 37 °C and shaken at 180 rpm (3 Hz) for 120 minutes. During the intestinal phase, the digesta were sampled at two-time points, after 20 minutes (T20) and after 120 minutes (T120). Ethanol at 96% (v/v) was used to stop the enzymatic reaction. The amount of digested starch was obtained from the concentration of glucose measured in the sample with the GOPOD assay kit from Megazyme (Bray, Ireland). Glucose absorbance was detected at 510 nm using Cary 60 UV – Visible spectrophotometer (Agilent Technologies, USA). The principal data obtained from Englyst's digestion were rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) (Englyst at el., 1996). RDS was the quantity of glucose released at 20 minutes of hydrolysis multiplied by 0.9 to convert this value into a starch amount. SDS

corresponded to the starch digested between 20 and 120 minutes of digestion. RS represented the difference between the total starch and the SDS. The units of RDS, SDS and RS were expressed in a gram of starch per 100 grams of total starch (% total starch on a wet basis). The analysis was repeated 12 times for each sample.

2.2.2.7 Differential scanning calorimetry (DSC)

The thermal properties and gelatinization behavior of flour, dough and bread were studied with the DSC Q2000 (TA instruments, Newcastle, NE, USA). The flour samples were wet with water (ratio 1:3, w/v), dough and bread were analyzed as they were. The different materials were weighted (~27 mg) and hermetically sealed into high volume pressure pans. The samples were heated at the rate of 5 °C/min from 2 ℃ to 150 ℃. The transition enthalpy (ΔH), the onset temperature (To), and the peak temperature (Tp) were analyzed using the software TA Universal Analysis (TA instruments, Newcastle, NE, USA) and expressed on a dry basis. The results were the mean of three measurements.

2.2.2.8 Confocal laser scanning microscopy (CLSM)

Cell walls and protein of each fraction of flour, dough and bread was observed using CLSM (Zeiss 510, Carl Zeiss microscopy, Oberkochen, Germany) and processed by Zen blue 2.3 edition software (Carl Zeiss microscopy, Oberkochen, Germany). The solution made with 0.01% Calcofluor white (CFW) and 0.005% Rhodamine B (RB) was used to stain the cell walls containing arabinoxylan and β-glucan in bluish color and simultaneously protein in purple gradation (Rovalino-Córdova et al., 2019). The three flours were dyed in culture tubes where 2 grams of samples were completed covered by the staining solutions. The incubation process was conducted overnight for both staining alternatives, without shaking. After that, samples were moved onto a

microscopical slide, elevated with a spacer. For the proofed dough, around 8 grams of each fraction was frozen in liquid nitrogen and kept at -18°C. After that, the samples were cut with a razor blade (Personna Verona, Italy) to remove surface irregularities and scrape off the outer layers of the dough. This resulted in samples with 3-5 mm thickness. Before staining, the samples were defrosted. The incubation with 2 mL of CFW-RB lasted 60 minutes. Bread was fixed with 2% agar in 0.1 M phosphate buffer pH 7.0 to preserve the structure and ease the cutting (1 x 2 x 2 mm) with a razor blade (Personna Verona, Italy). The slices of bread were stained with around 2 mL of dye and incubated overnight. CFW was excited by an argon laser at 405 nm and RB by He-Ne laser at 543 nm. The captured image was obtained from 10x or 20x magnification and a depth of 8 bits.

2.2.3 Data analysis

All data were presented as mean ± sample standard deviation (SD). Minitab18® Statistical Software (Pennsylvania, USA) was used to analyze the normality and differences of data between fractions, separately in flour, dough, and bread. Firstly, normal distribution was checked using Ryan-Joiner test ($p < 0.05$) and the skewness of z-value ($-1.96 < z < 1.96$). To confirm the assumption of equal variance, if the p-value was greater than 0.05 in Levene's test, then the difference in variance was not significant. For data with normal distribution and equal variance, one-way ANOVA ($p < 0.05$) was employed to determine the significance of differences and posthoc Tukey test to identify which sample was different from others. When the assumption of equal variance was not fulfilled, a post-hoc comparison was conducted using the Games-Howell method. In case major data were not normally distributed, the Kruskal Wallis test ($p < 0.05$) and post-hoc Mann-Whitney test ($p < 0.05$) was used.

2.3 Results

2.3.1 Proximate composition of flour

The small, medium and large flours were produced from the same grain sample and their proximate composition was similar (Table 2.2). The protein content ranged from 13.6% in SF to 11.9% in MF, without significant difference among the three flours. No significant differences were also found for the total starch, which content was around 47% on a dry basis. The damaged starch was detected only in the small flour. The moisture content was significantly lower in SF than in other samples.

Table 2.2 Proximate composition of flour samples obtained by durum wheat grain: small (< 350 µm), medium (> 1000 µm; < 1800 µm) and large (> 1800 µm) particle size.

	SF	**MF**	**LF**
Protein* **(%)**	13.6 ± 0.1[a]	11.9 ± 1.3[a]	12.5 ± 0.3[a]
Total starch (%)	54.1 ± 0.3[a]	55.2 ± 0.0[a]	53.6 ± 0.0[a]
Damage starch (%)	2.1 ± 0.1	n.d.	n.d.
Moisture (%)	6.9 ± 0.5[b]	8.4 ± 0.0[a]	8.0 ± 0.0[a]

*Protein conversion factor=5.83. Small flour (SF); medium flour (MF); large flour (LF). Values are expressed in mean ± SD in % of flour's weight on a wet basis. The same letter indicates no significant difference between mean values of the samples ($p < 0.05$, Tukey's test, n = 3).

2.3.2 Bread characterization

The bread production with the three flours was optimized to obtain doughs with the same consistency and the same CO_2 production during the leavening. Table 2.3 shows the results of bread characterization in terms of moisture, water activity and total starch, and the textural properties in terms of specific volume, hardness, and springiness. The moisture content of the large bread (LB) was significantly higher than the medium bread (MB) and small bread (SB), whereas that of MB and SB was comparable. Water activity was not significantly different in the three loaves. Moreover, no significant differences were found in the total starch content of bread, mirroring the values of the total starch content in the corresponding flours. As regards the textural properties, the specific volume significantly decreased when LF was used, but no significant difference was observed between SB and MB. An opposite trend was detected for the hardness, which is usually inversely correlated to the specific volume. LB had a significantly higher hardness compared to MB and SB, which instead were no significantly different. The high standard deviation of this analysis, especially in LB, was likely attributed to the irregular structure of the crumb. The springiness was significantly higher in bread made with SF than the other two bread produced with MF and LF.

Table 2.3. Characterization of bread made with durum wheat flour of different particle size (small < 350 µm, medium > 1000 µm; < 1800 µm and large > 1800 µm particle size).

	SB	MB	LB
Moisture (%)	42.7 ± 0.2^{b}	42.7 ± 0.2^{b}	45.1 ± 0.0^{a}
Aw	0.955 ± 0.001^{a}	0.959 ± 0.003^{a}	0.958 ± 0.002^{a}
Total starch (g/100 g_{bread})	33.1 ± 0.3^{a}	32.9 ± 0.4^{a}	32.6 ± 0.2^{a}
Specific volume (g/mL)	1.68 ± 0.01^{a}	1.66 ± 0.04^{a}	1.37 ± 0.07^{b}
Hardness (N)	30.8 ± 3.9^{b}	29.4 ± 6.5^{b}	55.1 ± 14.8^{a}
Springiness (-)	0.92 ± 0.01^{a}	0.88 ± 0.03^{b}	0.83 ± 0.09^{b}

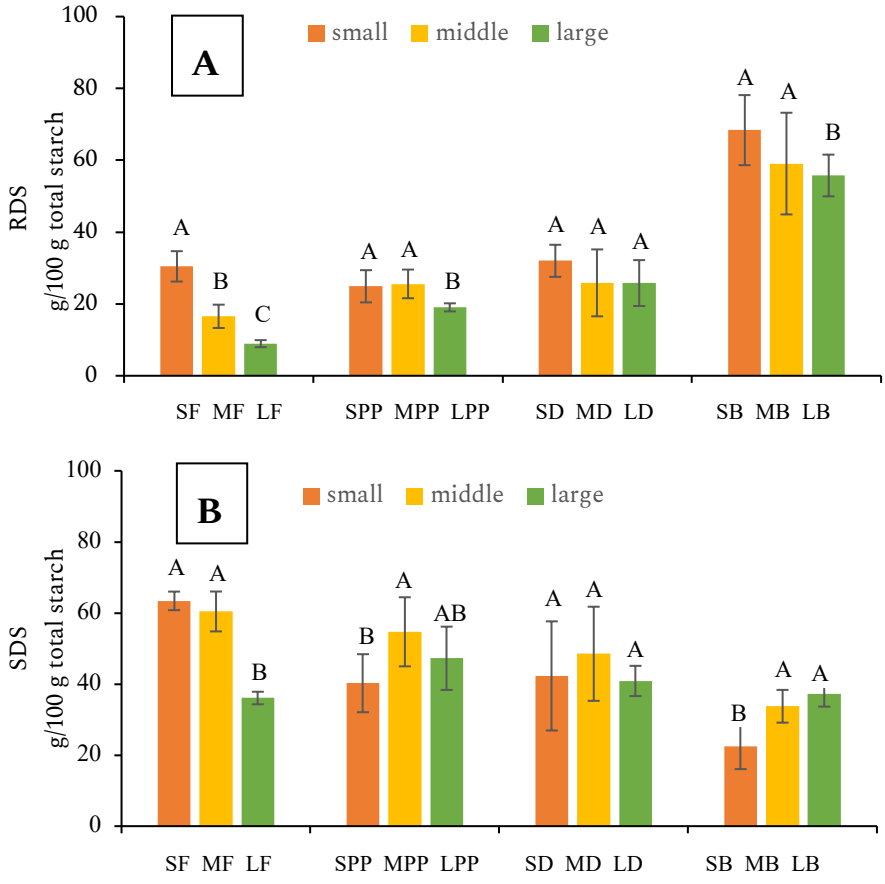
Values are expressed in mean ± SD. Small bread (SB); medium bread (MB); large bread (LB). The same letter within the same row indicates no significant difference between mean values ($p < 0.05$, Tukey's test, n =9).

2.3.3 Starch digestibility

RDS, SDS and RS of bread processing steps are presented in Figures 2.1a, 2.1b, 2.1c, respectively. The starch digestibility of pre-proofed dough was analyzed to study the effect of the fermentation step on the role of cell wall integrity in modulating starch digestibility. In flour samples, RDS significantly decreased, and RS significantly increased with the increase of granulometry. SDS content was similar in SF and MF but significantly lower in LF. Moreover, RDS was three times higher in SF than in LF and the RS nine times greater in LF than in SF.

The differences observed in starch digestibility among flours with different particle sizes became smaller in the pre-proofed doughs. RDS of LPP was significantly lower than that of MPP and SPP. SDS of MPP was significantly higher, whereas RS was significantly lower than the SPP. In proofed dough, these differences in starch digestibility among the three particle sizes disappeared meaning

particle size no longer had an effect in modulating the rate of starch digestibility. Instead, in bread, significant differences were again observed for RDS, SDS and RS among the three particle sizes. In LB, RDS was lower than that of SB and MB. SDS, instead, increased with the increasing of the particle size being significantly lower in SB. For the resistant starch, MB had the significantly highest value, followed by SB and LB.



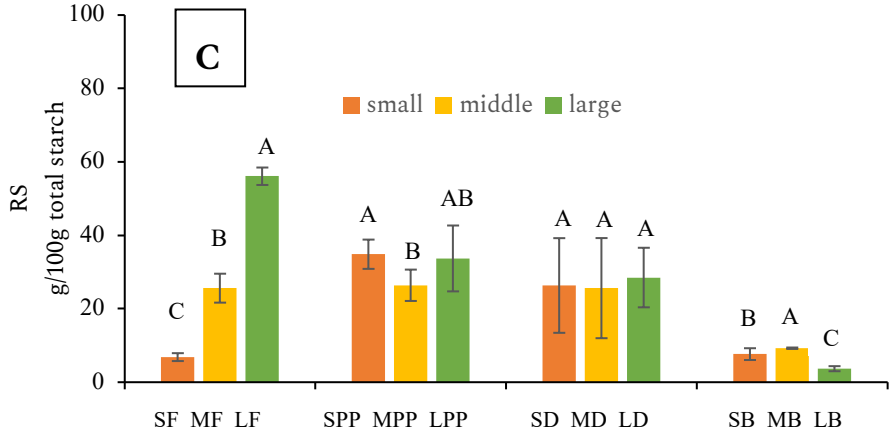


Figure 2.1. Rapid digestible starch (RDS) (panel A); slowly digestible starch (SDS) (panel B) and resistant starch (RS) (panel C) of flour, pre-proofed dough, dough and bread. Small flour (SF); medium flour (MF); large flour (LF); small pre-proofed dough (SPP); medium pre-proofed dough (MPP); large pre-proofed dough (LPP); small dough (SD); medium dough (MD); large dough (LD); small bread (SB); medium bread (MB); large bread (LB). The results are expressed as g/100 g total starch. The same letter indicates no significant difference among the three particle sizes for flour, pre-proofed dough, dough and bread ($p < 0.05$, Tukey's test, n =3).

2.3.4 Thermal properties

The thermal properties of flours, dough and bread made with the three particle sizes are shown in Table 2.4. The peak A starting at 45 °C in SB represents the melting energy of a small portion of retrograded starch. Retrograded starch, instead, was detected neither in MB and LB nor in flour and dough. The endothermic peak B, ranging around 57-69 °C and depending on the moisture content of the analyzed samples, represents the starch gelatinization in flour and the dough. This peak (B) was not present in bread because the starch was already gelatinized during the baking process. The peak C, which appeared at higher temperatures (~100 °C), is attributed to the energy needed to melt the amylose-lipid complexes. The flour starch gelatinization was not affected by the particle size of the flour, with none of the gelatinization parameters (To, Tp, and ΔH) being significantly different. For the peak C, the onset temperature of SF was significantly lower than that of MF and LF and the ΔH of SF was significantly higher than that of the other two flour particle sizes. In the dough, significant differences were found for both peaks. As shown in Table 2.4, SD had onset temperatures and transition enthalpies higher than the medium and large dough. In bread, the peak C was observed for all bread. For To, SB had the significantly lowest value, where the peak temperature increased with the increasing of the granule size, and for the enthalpy, SB showed the highest value, followed by the large and the medium bread.

Table 2.4. Thermal properties of flour, dough and bread made with small flour (< 350 µm), medium flour (> 1000 µm; < 1800 µm) and large flour (> 1800 µm).

	Peak A			Peak B			Peak C		
	T_o	T_p	ΔH	T_o	T_p	ΔH	T_o	T_p	ΔH
SF	-	-	-	57.1 ± 0.2^{a}	63.2 ± 0.1^{a}	6.0 ± 0.5^{a}	80.8 ± 1.2^{b}	92.9 ± 0.1^{a}	1.2 ± 0.1^{a}
MF	-	-	-	54.4 ± 0.6^{a}	59.8 ± 1.0^{a}	6.5 ± 1.2^{a}	87.7 ± 2.2^{a}	94.5 ± 0.0^{a}	0.6 ± 0.3^{b}
LF	-	-	-	55.4 ± 2.7^{a}	60.7 ± 2.4^{a}	6.1 ± 0.8^{a}	88.2 ± 1.0^{a}	94.7 ± 1.5^{a}	0.4 ± 0.1^{b}
SD	-	-	-	62.8 ± 0.2^{a}	69.6 ± 0.3^{a}	10.1 ± 0.4^{a}	122.2 ± 0.2^{a}	128.9 ± 0.1^{a}	0.7 ± 0.1^{a}
MD	-	-	-	60.9 ± 0.9^{b}	67.8 ± 1.0^{a}	7.5 ± 0.8^{b}	103.5 ± 0.7^{b}	111.7 ± 0.6^{b}	0.4 ± 0.1^{b}
LD	-	-	-	61.3 ± 0.3^{b}	68.2 ± 0.3^{a}	9.0 ± 0.8^{ab}	103.4 ± 0.5^{b}	112.0 ± 0.2^{b}	0.4 ± 0.1^{b}
SB	45.9 ± 1.0	59.6 ± 0.5	0.6 ± 0.1	-	-	-	88.3 ± 0.9^{b}	110.6 ± 0.5^{c}	2.6 ± 0.1^{a}
MB	-	-	-	-	-	-	98.5 ± 0.1^{a}	112.5 ± 0.2^{b}	1.2 ± 0.1^{c}
LB	-	-	-	-	-	-	97.7 ± 2.6^{a}	113.4 ± 0.1^{a}	1.6 ± 0.1^{b}

Values are expressed as mean ± SD. Onset temperature (To), peak temperature (Tp) and transition enthalpy (ΔH). Small flour (SF); medium flour (MF); large flour (LF); small dough (SDS); medium dough (MD); large dough (LD); small bread (SB); medium bread (MB); large bread (LB). The same letter within the same column indicates no significant difference among the three particle sizes, respectively, for flour, dough and bread. ($p < 0.05$, Tukey's test, n =3).

2.3.5 Confocal laser scanning microscopy

Confocal laser scanning microscopy images of flour, dough, and bread for all three particle sizes were shown in Figure 2.2. The cell wall appeared in a bluish color and simultaneously protein matters exhibited purple shade. In panels SF, SD and SB, the walls of the cells appeared partially or completely damaged. In panels MF, MD and MB, it is possible to visualize and detect a big cluster of intact cells in all the bread production steps. The pattern of the cell in MF resulted uniform and regular, in MD, instead, the cell shape is more rounded probably due to the hydration and consequent swallow of the wall. A big cluster of intact cells could also be observed for the large particle size in the micrographs representing flour, dough, and bread (panels LF, LM and LB). In the LF micrograph, the cells have different shapes: stretched, long and rectangular. This micrograph depicted the outer part of the endosperm where the pericarp starts.

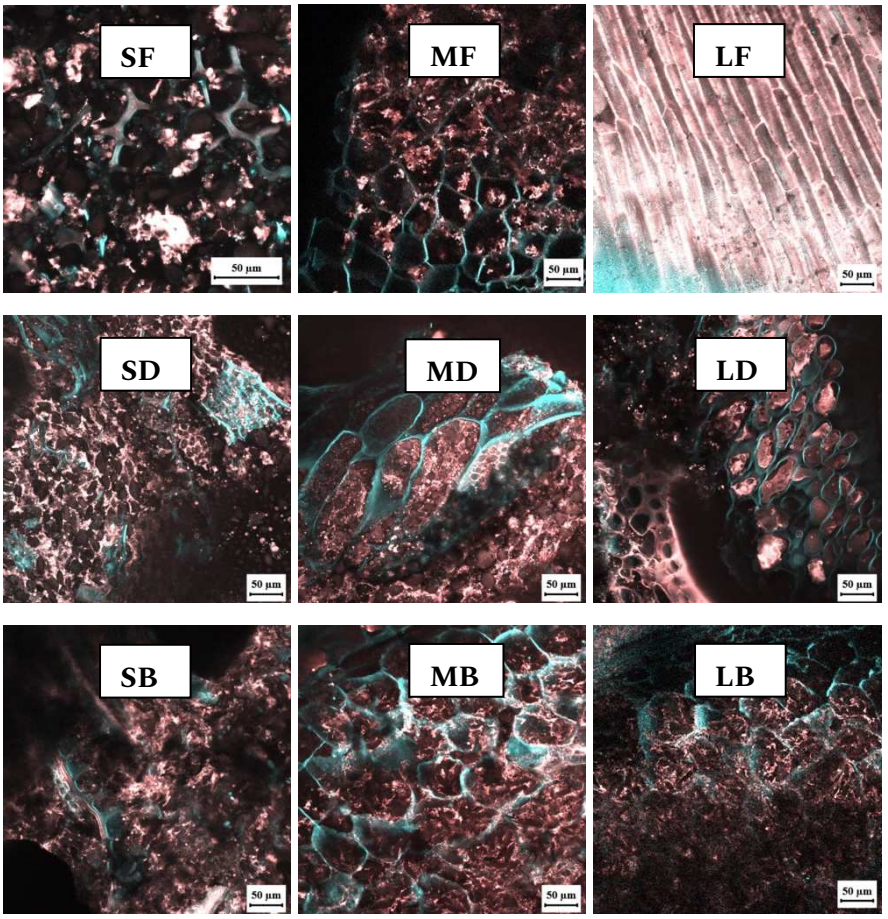


Figure 2.2. Confocal laser scanning microscopy images of small flour (SF); medium flour (MF); large flour (LF); small dough (SDS); medium dough (MD); large dough (LD); small bread (SB); medium bread (MB); large bread (LB). The samples were stained by Calcofluor White and Rhodamine B.

2.4 Discussion

The role of cell wall integrity in reducing the starch digestibility in cereals was intensely explored in the last ten years (Li, Chen et al., 2021). Preserving cell wall integrity, *e.g.*, increasing the particle size of the flour, has been suggested a promising and interesting way to decrease the starch digestibility in wheat flour and porridge but contradictory results have been reported when this strategy has been applied to bread. Therefore, in this work we investigated starch digestibility in flour with three particles sizes (i.e., small < 350 μm, medium-1000 - 1800 μm and large >1800 μm) as well as in dough and bread produced with those flours. Our results showed that the integrity of the cell wall was kept during the whole bread processing for the medium and large particle sizes whereas cell walls were mostly destroyed in the flour of small particle size. As expected, the effect of particle size on starch digestibility was observed in flour. Indeed, the starch digestibility was higher in SF than other flours and RDS, so the starch digested in 20 minutes was 21% higher in SF than in LF. This effect can be mainly ascribed to the presence of a higher fraction of intact cells in the flour of medium and large size since the three flours were comparable in terms of proximate composition. The only difference among flours was the presence of damaged starch (Table 2.2) and the high extent of the damage in the cell wall (Figure 2.2) in SF. These results confirm the findings of Bhattarai, et al., (2018) who studied the effect of cell wall intactness in modulating the starch digestibility of isolated cereal cells. The authors found that the cell wall, in the intestinal tract, acts as a barrier limiting the access of amylolytic enzymes to starch granules. The same findings were also reported by (Mandalari et al., 2018), who demonstrated that increasing particle size of porridge results in a decrease in starch digestibility.

As already demonstrated in previous studies (Edwards et al., 2015b; Guo et al., 2018), the enthalpy of gelatinization of starch was not affected by flour particle size, demonstrating that the observed effect

of cellular integrity on starch digestibility cannot be ascribed to changes in the thermal behavior of starch. However, the effect of particle size on starch digestibility was lost after the mixing and proofing step. The differences in starch digestibility observed in flour among the three particle sizes became smaller in the pre-proofed dough. A slight decrease in RDS by increasing particle size in pre-proofed dough still signals the contribution of increasing particle size of flour in reducing starch digestibility. However, in the dough, starch digestibility did not significantly differ among the different flours. This indicates that the effect of particle size was no longer able to modulate the starch digestibility even though, as depicted in the CLSM micrographs, intact cells were still present in the middle and large particle sizes. Therefore, we hypothesize that, during the mixing and fermentation steps, the porosity of the cell walls increased, and in turn increasing the accessibility of amylase to starch granules. The prolonged mixing and fermentation step could be responsible for the extended activity of the endogenous enzyme on arabinoxylans and β-glucans, which are the main components of the wheat cell wall (Andersson et al., 2004; Dornez et al., 2008). The activity of these enzymes, naturally present in the flour, could have increased the solubilization and structural redistribution of these components and the consequent increase of cell wall porosity.

RDS, SDS and RS in the bread digestion were significantly different among the three particle sizes but the magnitude of the effect was much smaller as observed in the flour. As explained before, big clusters of intact cells could be observed both in dough and bread. This is in line with the finding of (Korompokis et al., 2019), who detected intact cells in coarse flour (average particle size: 705 μm), even after a hydrothermal process, meaning that the swelling and the gelatinization of the starch were not able to destroy the cell integrity. Moreover, (Korompokis et al., 2021) found the intact cell walls were still detectable after the baking process, but, in this case, the integrity of the cell wall was not able to significatively decrease the digestibility,

as previously found in flour (Korompokis et al., 2019). This was probably due to the increased porosity of the cell wall during baking. It was previously demonstrated by (Cleemput et al., 1997) and (Comino et al., 2016) that the solubilization of arabinoxylans and β-glucans significantly increased during baking and this could have probably resulted in an increase in cell wall permeability. To explain the small difference in starch digestibility observed among the three breads we have looked at bread volume and texture. As shown in Table 3, bread made with large flour had a lower specific volume and subsequently the highest hardness among the samples. Generally, the bread volume is affected by two factors: the CO_2 production during the leavening and the ability of the viscoelastic gluten network to entrap and hold the gas during the proofing and the baking steps (Schober & Arendt, 2003). In our study, to better compare the three bread samples, the cumulative gas product during the proofing step was standardized, so the volume differences have to be mainly attributed to the gluten network strength. As previously shown by (Lin et al., 2020), the presence of bigger flour particles can inhibit the formation of a dense and structured network, through limiting protein crosslink. Moreover, (Bressiani et al., 2017) studied the effect of the particle size on the gluten density of dough made with flour with different granulometry. The authors demonstrated that finer flour formed denser gluten due to the greater interactions between the flour components. Therefore, it is possible to hypothesize that the higher volume found in small bread made with SF was probably due to the strong gluten network formed during the mixing. Conversely, bread made with large particle size was more compact and denser than the medium and small ones and this difference in the texture could have influenced the starch digestibility. (Martínez et al., 2018) already demonstrated that a compact structure of bread may decrease starch digestibility by limiting amylase access to starch. In our study, this dense and compact bread texture might have delayed the rate of starch digestion in the harder bread that was produced with the flour with the biggest size.

2.5 Conclusion

To settle the question on whether increasing the fraction of intact cells can reduce the GI of cereal-based products, in this study we monitored the modulating effect of durum wheat flour particle size on *in vitro* starch digestibility in each step of bread making. From our results, it is possible to conclude that the particle size and therefore, the fraction of intact cells could limit starch digestibility only in flour. During bread processing, the ability of the cell wall to limit the contact between starch and enzyme is lost, probably due to the increasing porosity of the cell wall or partial damage of the outer layer of the grain. The modest effect of particle size on starch digestibility in bread may be ascribed to crumb texture, i.e., a dense and compact structure in bread produced with large particle flour. In conclusion, this study shed further light on the effect of the bread matrix on starch digestibility and demonstrates that increasing flour particle size is not a suitable strategy to reduce GI of bread.

Role of particle size in modulating starch digestibility and textural properties in a rye bread model system

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Abstract

In cereal products, the use of flour containing clusters of intact cells has been indicated as a potential strategy to decrease starch digestion. Compared to wheat, rye possesses more uniform and thicker cell walls but its protective effect against starch digestion has not been elucidated. In this paper, rye flours of three different average particle sizes, i.e., large (LF), medium (MF) and small (SF), were used to produce a model bread, and *in vitro* starch digestibility and bread quality were studied. In MF (~1200 μm) and LF (~1700 μm), clusters of intact cells were present after bread processing; in SF (~350 μm), cell walls were damaged. Starch digestibility in flour was lower for LF and MF compared to SF. Instead, bread produced with MF and LF exhibited the least cohesive and resilient texture, disintegrated more during digestion, and exhibited the highest starch digestibility. The results highlight the central role of bread texture on *in vitro* starch digestibility.

Keywords:

Rye; bread model system; integrity of cell wall; cohesiveness; *in vitro* digestibility; confocal laser scanning microscopy.

3.1 Introduction

Rye (*Secale cereale* L.) is one of the major crops for bread production, second only to wheat (*Triticum aestivum* L.) (Arendt & Zannini, 2013; Deleu et al., 2020) even though it is considered a minor cereal represents 1% of total world cereal production (Arendt & Zannini, 2013). Rye has great resistance to cold temperatures and could be fruitfully bred in places with severe climates such as Germany, Poland, Russia, Denmark, and Belarus which together account for 85% of its total production (Arendt & Zannini, 2013; Kaur et al., 2021; Németh & Tömösközi, 2021).

Rye is utilized in the production of traditional foods, such as bread, pumpernickel, and flakes for porridges, or added to traditional wheat-based bakery products to reduce the starch digestibility and the consequent glucose absorption (Deleu et al., 2020). The consumption of rye products was proven to induce a low post-prandial insulin response, prolonged glucose profile, and increased satiety compared with wheat bread, and this effect was widely observed in several randomized controlled trials (Deleu et al., 2020; Jonsson et al., 2018). Different factors seem to be responsible for the beneficial effect of rye consumption. Firstly, rye is characterized by the greatest dietary fiber content among all cereals (Andersson et al., 2009). Soluble dietary fibers, which are present both in the rye bran and the endosperm, increase the digesta viscosity and, consequentially, decrease the extent of digesta mixing as well as enzyme diffusion. The digesta viscosity slows down the gastric emptying rate and, consequently, reduces intestinal carbohydrate digestion and absorption (Rosén et al., 2009). In addition, the presence of bioactive compounds, such as phenolic acids, could hinder carbohydrate hydrolysis, stimulate insulin secretion, and inhibit enzymatic activity (Jonsson et al., 2018; Rosén et al., 2011). Jenkins et al., (1986) also demonstrated the key role of the firm structure of rye bread (i.e., pumpernickel) in slowing down the glycemic response. Compared to wheat, rye gluten is less prone to

form a proper viscoelastic network due to the prolamin called 'secalins' which decreases the resistance to stretch, limiting the formation of an aerated and voluminous bread (Arendt & Zannini, 2013). In rye bread, indeed, the structure is formed by a continuous phase of starch granules embedded in a dense fiber matrix, mainly made by arabinoxylans. Therefore, the peculiar structure of rye bread, i.e., dense and compact, could reduce the physical disintegration during digestion and consequentially limit starch digestion and absorption at the intestinal level (Juntunen et al., 2003). In addition, when gluten is added to rye bread, a porous aerated structure is formed, the crumb easily disintegrates during digestion and, consequentially, its glycemic and insulin response results similar to the one elicited by refined wheat bread. This confirms the central role of the compact dense structure of rye bread in decreasing its starch digestibility (Nordlund et al., 2016).

In the last 10 years, the effect of cell wall integrity was deeply researched as a fruitful strategy to decrease starch digestibility, mainly in pulses where the cell wall is thick and poorly permeable (Bhattarai et al., 2018; Dhital et al., 2016; Rovalino-Córdova et al., 2019). Intact cell walls of pulses, made with undigestible polysaccharides, were reported to modulate the starch hydrolysis limiting the diffusion of α-amylase into the cell. The inhibition of enzyme diffusion into the cells could be ascribed mainly to the synergetic effect of small pores, naturally present in the cell wall, and the adsorption of enzymes on the surface of the wall (Rovalino-Córdova et al., 2021). For what concerns cereals, the intactness of the cell wall has been reported to be efficient in decreasing the digestibility of starch in isolated cells and flour from wheat, sorghum, and barley (Bhattarai et al., 2018; Korompokis et al., 2019; Lin et al., 2020). However, when wheat coarse flour, which contains clusters of intact cells, is used to produce bread, this effect is lost (Korompokis et al., 2021; Tagliasco et al., 2022). The authors hypothesized that this may be due to the increased porosity of the cell wall during bread processing due to the solubilization of

arabinoxylans and β-glucans, which could have enhanced contact between α-amylase and starch. Nevertheless, rye grains have been shown to possess a thicker cell wall than the wheat grain and the thickness of the walls is uniform in the different parts of the starchy endosperm, but it is not known yet whether this structural feature can be used to reduce the starch digestibility of rye bread and consequentially its GI (Autio & Salmenkallio-Marttila, 2001).

Against this background, rye flour varying in particle size was studied to elucidate the effect of intact cell clusters on the starch digestibility of rye flour and a model rye bread. Traditionally, rye bread is prepared by sourdough fermentation as the cell wall degradation and solubilization of pentosans and arabinoxylans promoted by acidic conditions is essential for rye bread quality (Arendt et al., 2007). However, it has been already demonstrated that the acidity produced by sourdough fermentation could have a direct effect in decreasing starch digestibility (De Angelis et al., 2009; Németh & Tömösközi, 2021). Therefore, to rule out the effect of sourdough fermentation on starch digestibility and cell wall degradation, a simple breadmaking process with baker yeast was used in this study. The textural quality, *in vitro* starch digestion, and physical disintegration during the digestion were further investigated to study the relationship among the integrity of cell walls, structural features of bread, and *in vitro* starch digestibility.

3.2 Materials and Methods

3.2.1 Materials

Rye (*Secale cereale L.*) grain was purchased from Tibiona (Villanova Mondovi, Italy). For the confocal laser scanning microscopy, Rhodamine B R6626 (≥95%, Sigma Aldrich) and Calcofluor White M2R (Fluorescence Bright 28, MP Biomedicals) were used as dyes. For the *in vitro* digestion, the following enzymes were used: pepsin P7000 (from porcine gastric mucosa, specific activity ≥ 250 units/mg solid); pancreatin P7545 (from porcine pancreas, 8 x USP); invertase I4504 (from baker yeast, specific activity ≥ 300 units/mg solid); and amyloglucosidase A7095 (from *Aspergillus niger*, ≥ 260 U/mL) (all from Sigma Aldrich). The other chemicals and solvents utilized were of analytical grade.

3.2.2 Methods

3.2.2.1 Flour preparation

The rye was ground using a multi-mill (Alpine Hosokawa, Augsburg, Germany). The following conditions were set up: speed: 1700 rpm (28.33 HZ); power: 500 W; difference pressure: 30.0 mbar; airflow, 52 m^3/h. After milling, two meshes were used to sieve the flour: 1800 µm and 1000 µm. The flour retained on the 1800 µm sieve was codified as large flour (LF), and the fraction between 1000 and 1800 µm was identified as medium flour (MF). To obtain a small particle size, a certain quantity of large flour was milled again following these conditions: speed: 14000 rpm (233.33 Hz); power: 600 W; pressure difference: 30.0 mbar; airflow, 52 m^3/h. This re-milled fraction was named from now on as a small flour (SF).

3.2.2.2 Flour characterisation

The particle size distribution (PSD) of the three different rye samples was determined by sieving the flours through 9 sieves with decreasing mesh diameters: 2500 µm, 2000 µm, 1400 µm, 1000 µm, 800 µm, 500 µm, 300 µm, 150 µm, 50 µm. A representative amount of sample (100 g) was poured inside the sieve column placed in an electromagnetic shaker (Vibra Filtration, Barcelona, Spain), and shaken for five minutes. The analysis of PSD was repeated five times for each flour and the results were expressed as the percentage of mass particles retained on each sieve. The moisture content of the flour was measured following the official method (Method AACC 44-15.02., 1999). Total starch content and starch damage were analyzed following the same method utilized by Tagliasco et al. (2022). Before the determination, LF and MF were re-milled using Freezer/Mill 6875D (Spex SamplePrep, USA) to produce a final particle size < 500 µm as recommended in the kit protocol. Starch damage was determined using a Starch Damage kit (Megazyme, Bray, Ireland). The resulting glucose was detected by a colorimetric method and the absorbance was measured at 510 nm using a spectrophotometer Cary 50 (Agilent Technologies, USA). The amount of protein contained in flour was determined using the Dumas method with a flash EA 1112 NC analyzer (Thermo Fisher Scientific Inc., Waltman, United States of America) following the protocol given by the manufacturer. The conversion factor for rye protein was 5.83 (Müller, 2017). Soluble and insoluble fiber was analyzed according to Megazyme kit, K-TDRF (Megazyme, Wicklow, Ireland). The medium and the large samples were previously milled to pass through a sieve with a mesh of 500 µm. Three replicates on three different days were done for all the measurements.

3.2.2.3 Bread model system preparation

A bread model system made up of rye flour, water, salt, and yeast (*Saccharomyces cerevisiae*), as displayed in Table 3.1, was used to produce rye bread. The optimum of water and the mixing time to obtain the final dough consistency of 420 Brabender Units (BU) were studied with a water absorption test at 30 ºC and 63 rpm using a Farinograph (Brabender GmbH & Co KG, Duisburg, Germany) following the same method previously described (Tagliasco et al., 2022). The ingredients were mixed at 20 °C with a Hobart mixer (N50, Hobart, Woerden, The Netherlands). After the mixing step, the dough was portioned into 5 loaves weighing approximately 90 g and transferred into baking tins (Patisse, bread form 9 cm). The loaves were placed in an incubator at 30 °C and 75% humidity. *Saccharomyces cerevisiae* was used as a leavening agent instead of sourdough, commonly used for rye bread production, to rule out the effect of acid production on bread digestibility (De Angelis et al., 2009). The leavening time for each sample was optimized using a Risograph (National Manufacturing Co, Lincoln, Nebraska, USA) and the software used to analyze the data was Risosmart (National Manufacturing C., Lincoln, Nebraska, USA). The bread samples were placed at 30 °C/760 bar in a controlled temperature and humidity chamber SDCC-1P/W (Koma koeltechnische Industrie B.V, Roermond, The Netherlands). The proofing time for SF was set as 90 minutes and the proofing time for the MF and LF was established to make the same amount of cumulative CO_2 as that produced by SF in 90 minutes. The formulation for each particle size bread is displayed in Table 1. After the baking step, the loaves were removed from the tins and cooled down on a rack for 1 h. Three out of the 5 bread were kept in a Ziploc ® bag 17.7 x 18.8 cm (SC Johnson, United States of America) overnight to perform the quality measurements the day after. The other two bread loaves were frozen to measure the digestibility and to further observe the microstructure with confocal laser scanning microscopy. Bread production was repeated three times. Three

variations in formulations were studied: a bread produced with SF (coded SB), one with MF (coded MB), and one obtained with LF (coded LB).

Table 3.1. Formulation of rye bread made with bread made with SF = flour made with small particle size; MF = flour made with medium particle size; LF = flour made with large particle size. Ingredients are expressed on 100 g of flour.

	Flour (g)	**Fast action yeast (g)**	**Salt (g)**	**Water (g)**	**Mixing time (min)**	**Proofing time (min)**
SB	100	2	2	62	5	90
MB	100	2	2	60	60	90
LB	100	2	2	59	90	95

3.2.2.4 Bread quality characterization

The moisture content of around 3 g of breadcrumbs was analyzed using the quoted official method (Method AACC 44-15.02., 1999). Total starch of bread samples was analyzed following the instructions reported in the assay kit TSTA purchased by Megazyme (Bray, Ireland). The procedure followed for the analysis was the determination of the total starch content of samples containing resistant starch (RTS-NaOH Procedure -Recommended). The soluble and insoluble fiber was determined using the enzymatic method K-TDRF, (Megazyme, Wicklow, Ireland). For the protein determination, the Kjeldal method was used following the manufacturer instructions. The conversion factor for rye protein was 5.83 (Müller, 2017). The volume of each loaf was measured by the rapeseed displacement method following the official analytical protocol (Method AACC 10-05.01, 2009). The specific volume was expressed as the proportion between volume and weight of the sample (cm^3/g). The water activity of bread samples was measured at 25 °C utilizing an a_w-meter

(LabMaster-aw, Novasin, Lachen, Switzerland). Briefly, around 2 g of breadcrumb was let equilibrate at 25 °C in the a_w-meter and then the a_w was measured. Representative images of whole bread and the respective slices were captured using an image acquisition cabinet (Immagini & Computer, Bareggio (Milan), Italy) which was equipped with a digital camera (EOS 550D, Canon, Milan, Italy). The textural properties of bread were measured using a TA-XT plus analyzer (Stable Micro Systems, Godalming, UK) following the method previously described (Tagliasco et al., 2022). The analyzed parameters were hardness (N), the force required to deform the food in the first bite (Lapčíková et al., 2019); cohesiveness (-), the extent to which bread deforms when compressed and resilience (-), the ability of the sample to regain its original height (Lapčíková et al., 2019). The analysis was carried out in triplicate.

3.2.2.5 *In vitro* starch digestibility of flour and bread

The digestibility of rye flour and bread was determined according to *in vitro* Englyst's method (Englyst et al., 2018). Briefly, 2 g of sample was weighed, the flour as it was, and the bread was cut into cubes of about 5 x 5 x 5 mm^3. The *in vitro* digestion procedure is divided into two phases (i.e., gastric one lasting 30 minutes and intestinal one lasting 120 minutes). In the first phase, the samples were mixed with 10 mL of guar gum solution (0.05 M HCl) with pepsin (≥ 250 units/mg solid) and were shaken for 30 minutes at 37 ºC and 180 rpm. During the intestinal phase, 5 mL of enzyme solution containing pancreatin (8 x USP), invertase (≥ 300 units/mg solid), and amyloglucosidase (≥ 260 U/mL), have been added to the digesta together with 10 mL of 0.25 M sodium acetate buffer (37 ºC) and 5 marbles. The samples were kept at 37 °C for 120 minutes and agitated at 180 rpm. At minutes 20 and 120, 100 µL of digesta were sampled and the enzymatic reaction was stopped with the addition of 4 mL of ethanol 96 %. Later samples were centrifuged at 14000 rpm, 50 µL was sampled, and let react for 20

minutes at 50 °C with 1.5 mL of GOPOD reagent (Megazyme, Bray, Ireland). The spectrophotometer Cary 60 UV – Visible (Agilent Technologies, USA) was used to measure the absorbance at 510 nm.
The data obtained from Englyst's digestion were used to calculate the rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS). RDS represents the amount of glucose released in the first 20 minutes of digestion multiplied by 0.9 to convert the glucose detected into a starch amount. SDS represents the quantity of glucose released between minutes 20 and 120. RS represents the fraction of starch that was not digested after 120 minutes and was calculated as the difference between the total starch (TS) and the digested starch (RDS+SDS). Total starch content was measured following the kit protocol Total Starch assay (Megazyme, Bray, Ireland). The data obtained, RDS, SDS and RS were expressed in grams of starch per 100 g of total starch on a wet basis. The total starch content was measured 6 times for each bread and the *in vitro* digestibility was performed 12 times for each sample.

3.2.2.6 The disintegration of the sample during in *vitro* digestion

The disintegration of the samples during the *in vitro* digestion was studied by image analysis, measuring the particle size of the digesta over time. Briefly, 2 g of each bread sample was prepared as described in the previous paragraph (2.2.5) and subjected to *in vitro* digestion. The particle size of the digesta was measured after the gastric phase (T0) and at T20 and T120 of the intestinal phase. At these time points, the total digesta was diluted with 40 mL of water, placed in a flat plastic container (20.3 cm x 30.5 cm x 5.1 cm), and gently spread with a spatula. The images of the digesta were captured by CanoScan 9000 F MarkII (Canon Europa, Amstelveen, The Netherlands), and analyzed by ImageJ (ImageJ, Bethesda, Maryland, USA) following the method described by Chen et al., (2021). The captured images were transformed in 8-bit, adjusted for brightness/contrast, with a

threshold of 50-255. For each image, particles smaller than 0·015 mm^2 were removed from data analysis to prevent influence with the background. From each measurement, the areas (mm^2) of the digesta particles were collected and categorized in 7 intervals mm^2; **1**: < 0.12; **2**: ≥ 0.12, <0.3; **3**: ≥ 0.3, < 0.6; **4**: ≥ 0.6, < 0.9; **5**: ≥ 0.9, < 1.2; **6**: ≥ 1.2, < 1.5; **7**: ≥1.5 to show the particle size distribution in the different phase of *in vitro* digestion (Suo et al., 2021). All measurements were done in quadruplicate.

3.2.2.7 Confocal laser scanning microscopy (CLSM)

CLSM Stellaris 5 (Leica Microsystem CMS GmbH Wetzler, Germany) was used to detect the cell wall integrity in each particle size in rye flour and bread. The software Leica Application Suite X (LAS X) (Leica Microsystem CMS GmbH Wetzler, Germany) was used to process and analyze the micrographs. For flour, around 2 g of sample was weighed in a glass tube and stained with around 2 mL of a solution containing 0.005% Rhodamine B (RB) and 0.01% Calcofluor white (CFW) for one hour. The cell walls, consisting mainly of arabinoxylan and β-glucan, were stained in blue with CFW, and at the same time, the proteins were stained in red/purple color with RB (Rovalino-Córdova et al., 2019). After that, the flour was placed on a glass slide, elevated with a spacer, and analyzed. Regarding bread, it was defrosted 16 h before the measurement and carefully cut with a razor blade (Personna, Verona, Italy) to obtain slices 1 mm thick and stained in the same conditions already explained for flour. RB was excited at 543 nm with He-Ne laser and CFW at 405 nm with argon laser. The pictures were captured at two levels of magnification (i.e., 10x or 20x) and a depth of 8 bits.

3.2.3 Statistical analysis

The data were expressed as mean ± standard deviation (SD) and subjected to statistical analysis by IBM SPSS Statistics for Windows version 23.0 (IBM Corp., Armonk, N.Y., USA). The statistical method used to determine the significance differences between the analyzed samples was a One-way ANOVA ($p \leq 0.05$) and a post-hoc Tukey's test ($p \leq 0.05$) was performed to identify which sample was different from the others. The chi-square test ($p \leq 0.05$) was used to assess the statistical differences between the particle size distribution of the digesta of SB, MB and LB, for each time point. The analysis was performed in the XLSTAT version 2021.1 software.

3.3 Results

3.3.1.1 Flour characterization

The particle size distribution of the three flour samples is displayed in Figure 3.1. SF, obtained by double milling LF, had a particle distribution ranging from 0 to 500 µm, with a peak at 150 µm. MF ranged between 800 µm to 2000 µm, and 70% of the particles were bigger than 1000 µm and smaller than 1400 µm. LF instead, presented 80% of the particles between 1400 µm and 2000 µm. The proximate composition of the flour fractions is shown in Table 3.2. LF, MF, and SF were not different in terms of protein, total starch content and soluble and insoluble dietary fiber. However, the moisture content of SF was significantly lower than that of MF and LF. Damaged starch damage was detected only in SF since it underwent a double milling process.

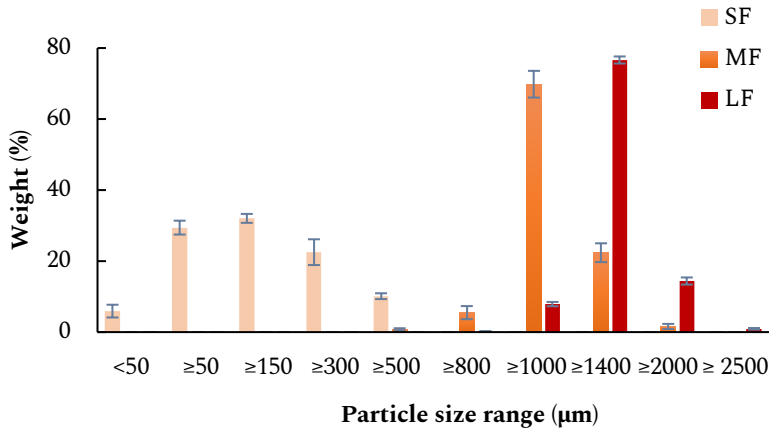


Figure 3.1. Particle size distribution (PSD) of rye flours. SF = flour made with small particle size; MF = flour made with medium particle size; LF = flour made with large particle size. Results are expressed as mean particle weight retained on each sieve % ± SD: 2500 µm, 2000 µm, 1400 µm, 1000 µm, 800 µm, 500 µm, 300 µm, 150 µm, 50 µm

Table 3.2. Protein, total and damaged starch, moisture content, and soluble and insoluble dietary fiber of rye flour with different particle sizes.

	protein content (g/100 g)*	**total starch (g/100 g)**	**damaged starch (g/100 g)**	**moisture content (g/100 g)**	**soluble fiber (g/100 g)**	**insoluble fiber (g/100 g)**
SF	7.94 ± 0.18[a]	51.58 ± 3.32[a]	0.70	9.95 ± 0.33[a]	3.72 ± 0.34[a]	11.83 ± 0.40[a]
MF	7.99 ± 1.11[a]	49.07 ± 4.22[a]	n.d.	11.97 ± 0.48[b]	3.21 ± 0.54[a]	12.84 ± 0.97[a]
LF	8.04 ± 2.64[a]	46.36 ± 0.74[a]	n.d.	12.12 ± 0.32[b]	3.18 ± 0.84[a]	11.99 ± 1.87[a]

Values are reported as % of flour's fresh weight and expressed as mean ± SD. Mean values (n=9) within a column with different letters were significantly different (p<0.05; Tukey's test). *Protein conversion factor = 5.83; "n.d.": not detected; SF = flour made with small particle size; MF = flour made with medium particle size; LF = flour made with large particle size.

3.3.2 Bread characterization

The characteristics of breads made with different particle sizes and their textural properties are shown in Table 3.3 and Table 3.4, respectively. The production of bread was standardized to have the same consistency after the mixing step and the same amount of CO2 produced during the leavening. However, significant differences in bread properties were found among the three samples. The moisture content of MB was significantly higher than that of SB and LB, whereas no difference was observed between SB and LB (Table 3.3). The water activity showed a different pattern, the lowest value was found for SB, whereas MB and LB were not significantly different (Table 3.4). Water activity was well related to moisture content, which is logical for this set of samples. For what concern the total starch, soluble and insoluble dietary fiber, no significant differences were found among the three samples (Table 3.3). The protein content instead increased, increasing the particle size of the bread. Moreover, the volume of bread decreased with increasing particle size. Indeed, the volume of SB was statistically higher than the other samples, whereas there was no significant difference between MB and LB. The values of hardness were inversely correlated to the specific volume (Table 3.4). SB showed the lowest value, instead, MB and LB had similar hardness. Cohesiveness and resilience decreased with the increase of the particle size. Representative images of the entire bread loaf and slices were shown in Figure 3.2, and they clearly displayed the difference in structure among the three bread samples prepared with increasing particle sizes. The crumb of SB was quite uniform, compact, and cohesive, however, the crumb of MB and LB appeared fragile, non-homogeneous, and easy to break. Moreover, in MB and LB the flour particles were still visible, and they were not well embedded in the dough structure as in SB.

Table 3.3. Moisture content, total starch, soluble and insoluble dietary fiber and protein of rye bread samples produced with small particle size flour, medium particle size flour, and large particle size flour.

	moisture content (g/100 g)	**total starch (g/100 g)**	**soluble fiber (g/100 g)**	**insoluble fiber (g/100 g)**	**protein (g/100 g)**
SB	41.8 ± 0.6^{b}	33.3 ± 2.1^{a}	2.66 ± 0.58^{a}	7.74 ± 0.07^{a}	4.73 ± 0.21^{a}
MB	43.1 ± 0.5^{a}	31.7 ± 2.7^{a}	2.16 ± 0.62^{a}	8.11 ± 0.05^{a}	4.96 ± 0.18^{ab}
LB	42.5 ± 0.5^{b}	30.3 ± 0.5^{a}	2.12 ± 0.22a	8.59 ± 0.60^{a}	5.26 ± 0.33^{b}

Values are reported on fresh weight and expressed as mean ± SD. Mean values (n=9) within a column with different letters were significantly different (p < 0.05; Tukey's test). SB = bread made with small particle size flour; MB = bread made with medium particle size flour; LB = bread made with large particle size flour.

Table 3.4. Water activity, specific volume, hardness, cohesiveness and resilience of rye bread samples produced with small particle size flour, medium particle size flour, and large particle size flour.

	water activity	**specific volume (cm^3/g)**	**hardness (N)**	**cohesiveness**	**resilience**
SB	0.95 ± 0.00^{b}	1.27 ± 0.04^{a}	50.3 ± 4.2^{b}	0.57 ± 0.03^{a}	0.26 ± 0.02^{a}
MB	0.96 ± 0.00^{a}	0.98 ± 0.04^{b}	61.8 ± 8.9^{a}	0.45 ± 0.03^{b}	0.22 ± 0.02^{b}
LB	0.96 ± 0.00^{a}	1.01 ± 0.04^{b}	58.3 ± 5.3^{a}	0.38 ± 0.05^{c}	0.17 ± 0.03^{c}

Values are expressed as mean ± SD. Mean values (n=9) within a column with different letters were significantly different (p< 0.05; Tukey's test). SB = bread made with small particle size flour; MB = bread made with medium particle size flour; LB = bread made with large particle size flour.

Figure 3.2. Representative images of the whole bread samples (A, B, C) and the respective slices (D, E, F) of SB = bread made with small particle size flour; MB =bread made with medium particle size flour; LB = bread made with large particle size flour.

3.3.3 Starch digestibility

The results of *in vitro* starch digestion of rye flour and rye bread are shown in Figure 3.3. Regarding the flour starch digestibility, RDS, SDS, and RS values of SF were significantly different from those of MF and LF, which were not significantly different. MF and LF had a lower value of RDS and SDS than SF, whereas SF showed the lowest value for RS. Therefore, the amount of glucose released between minutes 20 and minutes 120 during enzymatic hydrolysis (SDS) was higher in SF than in the other flours. When the flours were used for breadmaking, significant differences were found for SDS, where SB presented the lowest values, followed by LB and MB, which were not significantly different. Moreover, RS in bread had an opposite trend compared to

that found in flour, being SB the bread with a significantly higher value of RS than other bread samples. No difference, instead, was found in RDS among bread samples.

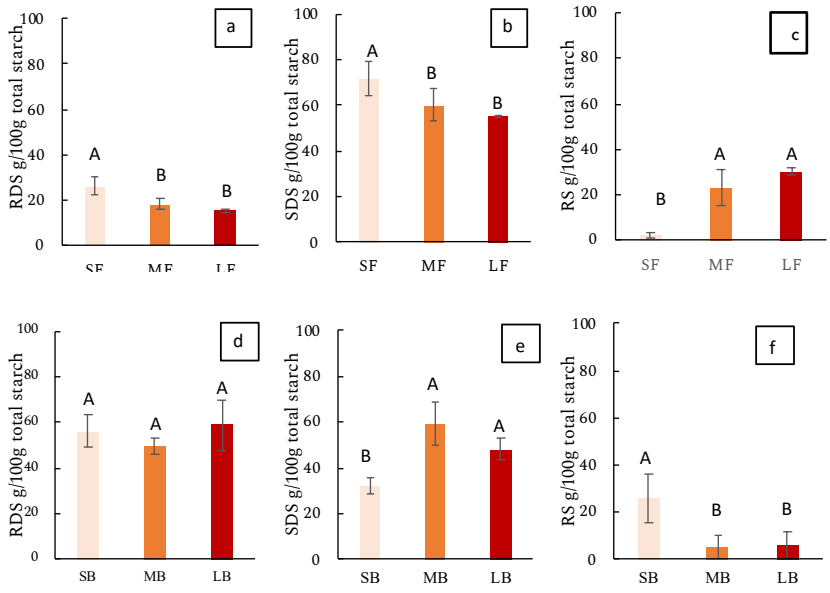
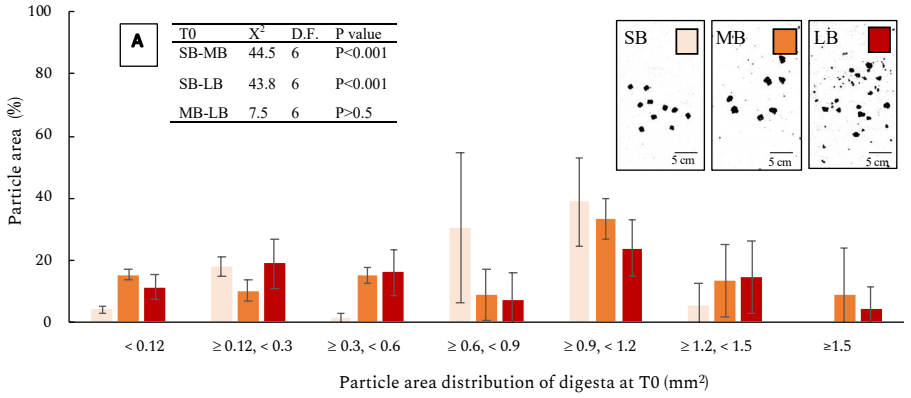
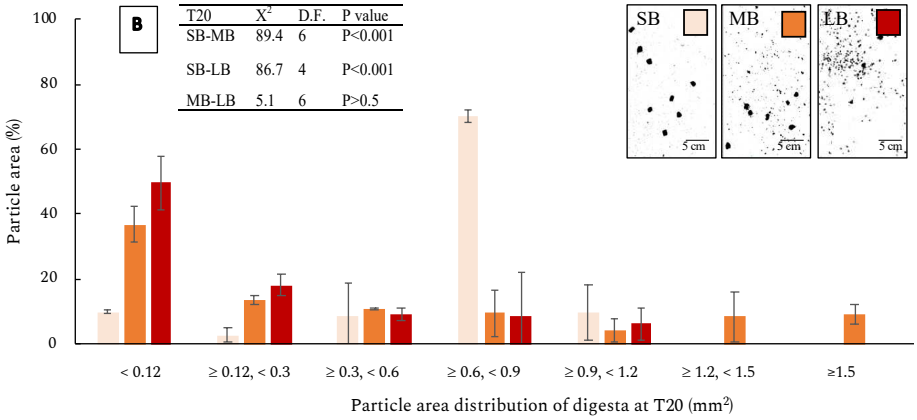


Figure 3.3. Rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) of rye flours with different particle sizes (panels a, b, and c) and of rye bread made with flours with different particle sizes (panels d, e and f). SF = flour made with small particle size; MF = flour made with medium particle size; LF = flour made with large particle size; SB = bread made with small particle size flour; MB =bread made with medium particle size flour; LB = bread made with large particle size flour. Results are expressed as % of the total starch. Columns sharing the same letter were not significantly different ($p< 0.05$; Tukey's test) (n=9)

3.3.4 Disintegration of bread sample during in *vitro* digestion

To understand how bread samples were disintegrated during the *in vitro* digestion, the images of digested particles were captured after the gastric phase (T0) (Figure 3.4 panel a), at 20 minutes (T20) (Figure 3.4 panel b), and the end of the intestinal phase (T120) (Figure 3.4 panel c). The mean area of the particles decreased from T0 to T120, for all three samples, but the magnitude of these differences changed among the three bread loaves made with increased flour particle size. As shown in Figure 3.4a, at T0, the digesta from sample SB showed a normal distribution with a peak in the interval around 0.9-1.2 mm^2. The digesta particles of MB, instead, were largely distributed among 7 intervals, with a peak around 0.9-1.2 mm^2. For the LB, two populations were observed, the first peaking in the interval between 0.12-0.3 mm^2 and the second one at 0.9-1.2 mm^2. At T20 (Figure 3.4 panel b), instead, the particles of SB were significantly bigger than those of both LB and MB. Seventy per cent of the SB particles were around 0.6-0.9 mm^2, instead, 50% of particles of MB and LB had a size smaller than 0.3 mm^2. At the end of the *in vitro* digestion (Figure 3.4 panel c), 80% of SB and MB particles were smaller than 0.12 mm^2, instead, for LB, 30% of them ranged between 0.12 and 0.3 mm^2, probably due to cell clusters, bigger in LB than SB and MB.


A
T0 X² D.F. P value
SB-MB 44.5 6 P<0.001
SB-LB 43.8 6 P<0.001
MB-LB 7.5 6 P>0.5
SB
MB
LB
5 cm
Particle area (%)
100
80
60
40
20
0
< 0.12
≥ 0.12, < 0.3
≥ 0.3, < 0.6
≥ 0.6, < 0.9
≥ 0.9, < 1.2
≥ 1.2, < 1.5
≥1.5
Particle area distribution of digesta at T0 (mm²)


B
T20 X² D.F. P value
SB-MB 89.4 6 P<0.001
SB-LB 86.7 4 P<0.001
MB-LB 5.1 6 P>0.5
SB
MB
LB
5 cm
Particle area (%)
100
80
60
40
20
0
< 0.12
≥ 0.12, < 0.3
≥ 0.3, < 0.6
≥ 0.6, < 0.9
≥ 0.9, < 1.2
≥ 1.2, < 1.5
≥1.5
Particle area distribution of digesta at T20 (mm²)

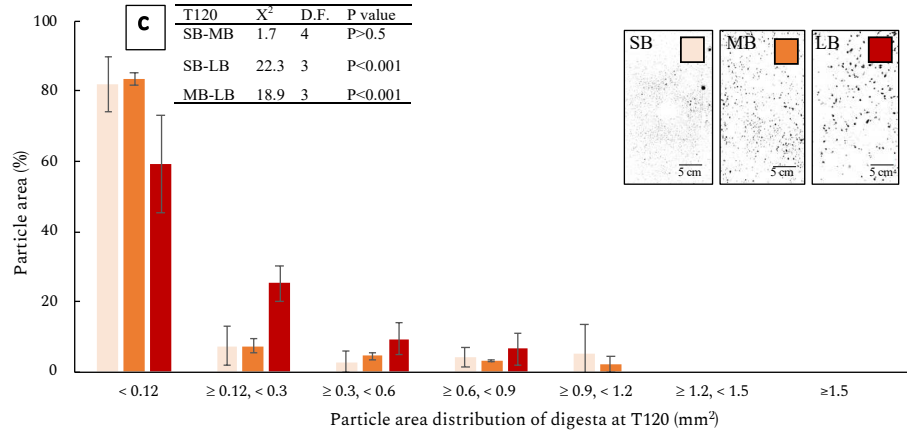

C
T120 X² D.F. P value
SB-MB 1.7 4 P>0.5
SB-LB 22.3 3 P<0.001
MB-LB 18.9 3 P<0.001
SB
MB
LB
5 cm
Particle area (%)
100
80
60
40
20
0
< 0.12
≥ 0.12, < 0.3
≥ 0.3, < 0.6
≥ 0.6, < 0.9
≥ 0.9, < 1.2
≥ 1.2, < 1.5
≥1.5
Particle area distribution of digesta at T120 (mm²)

Figure 3.4. Representative images (in the upper right corner) and particle size distribution of digesta particles after the gastric phase (T0, panel a), after 20 minutes of intestinal phase (T20, panel b), and at the end of the intestinal phase (T120, panel c) of rye bread samples. SB = bread made with small particle size flour; MB =bread made with medium particle size flour; LB = bread made with large particle size flour. Particle size distribution of digesta divided into 7 intervals mm^2; **1**: < 0.12; **2**: ≥ 0.12, < 0.3; **3**: ≥ 0.3, < 0.6; **4**: ≥ 0.6, < 0.9; **5**: ≥ 0.9, < 1.2; **6**: ≥ 1.2, < 1.5; **7**: ≥ 1.5. P value < 0.05 in chi squared test showed significantly differences between the digesta distribution: X2 = Chi squared value, D.F. = degree of freedom.

3.3.5 Confocal laser scanning microscopy

CLSM was used to capture images of rye flour and bread, as shown in Figure 3.5. The samples were stained to simultaneously visualize the cell wall (in blue) and protein (in red). The cell walls in sample SF appeared to be extensively broken into small pieces (Figure 3.5A), likely because of double milling. Instead, a significant amount of intact cell clusters was observed in samples MF and LF (Figures 3.5B and 3.5C). A similar pattern was observed in bread samples. Cell walls appeared to have completely lost their integrity in sample SB (3.5D), whereas big clusters of intact cells were still detectable in samples MB and LB (3.5E and 3.5F). The shape and the size of the cells seemed not to have been radically affected by bread processing.

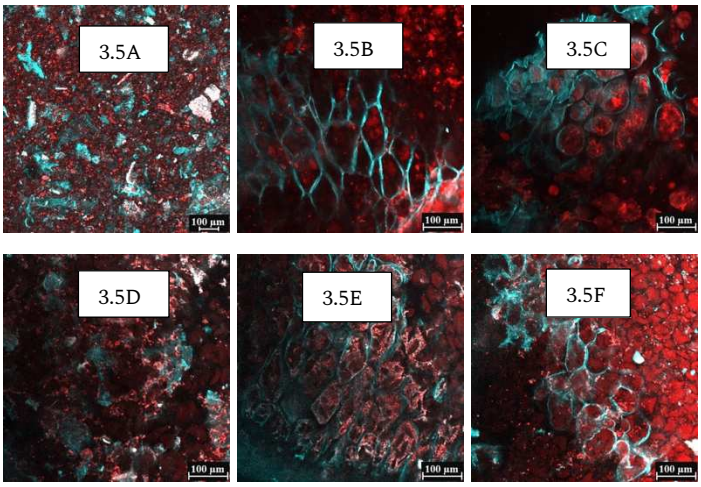


Figure 3.5. Representative confocal laser scanning microscopy images of SF = flour made with small particle size (3.5A); MF = flour made with medium particle size (3.5B); LF = flour made with large particle size (3.5C); SB = bread made with small particle size flour (3.5D); MB =bread made with medium particle size flour (3.5E); LB = bread made with large particle size flour (3.5F). Calcofluor White (0.01%) and Rhodamine B (0.005%) were used as dyes to stain simultaneously the cell wall (light blue) and the protein (red).

3.4 Discussion

The effect of cell integrity of plant tissues on starch digestibility has been extensively studied in the last decade in different pulses and cereals (Bhattarai et al., 2018; Dhital et al., 2016). However, to the best of our knowledge, this effect has not been studied in rye, even if this grain is characterized by a thicker cell wall than wheat grain (Autio & Salmenkallio-Marttila, 2001). In the present study, the effect of three different particle sizes on the digestibility and textural quality of rye flour and bread produced therefrom were investigated.

CLSM images revealed the presence of large clusters of intact cells in MF and LF, and their integrity was maintained during bread processing, i.e., MB and LB. Instead, the cell walls in SF and SB were largely damaged due to the harsh milling conditions. The starch digestibility of rye flour was inversely correlated with the particle size of the three fractions: increasing the particle size, the starch digestibility decreased. The effect of particle size on cell wall integrity and starch digestibility has been previously observed in legumes (Bhattarai et al., 2017; Dhital et al., 2016; Rovalino-Córdova et al., 2019) and cereals like wheat, sorghum and barley (Bhattarai et al., 2018; Korompokis et al., 2019; Lin et al., 2020). In LF, starch is mostly protected by intact cell walls, which restricts the α-amylase enzyme diffusivity inside the particles and acts as a barrier limiting its contact with starch. In contrast, the physical hindrance is lost once the structure of the grain is damaged by the milling process to transform it into fine flour so that starch can be easily digested.

In bread, instead, the results obtained on starch digestibility were quite unexpected. RDS, which represents the amount of starch digested after 20 minutes, was not significantly different among the three bread samples. Instead, SDS was significantly lower in SB than in MB and LB. Moreover, SB contained the highest amount of RS among the three analyzed bread. Overall, the extent of starch digestibility in bread made with SF was lower than in bread made with

bigger particle sizes and, therefore, with clusters of intact cells. The physical hindrance effect in MF and LF is lost in the corresponding bread, despite intact cell walls being clearly detected in the CLSM images. Similar behavior was recently observed for wheat where the effect of particle size on starch digestion was lost when the flours were processed into breads (Korompokis et al., 2021; Tagliasco et al., 2022). These authors showed that during bread processing the cell wall was not damaged, but its porosity increased due to the change in molecular weight of arabinoxylans. This seems to have occurred also in bread produced from rye grain, even though rye was reported to have a thicker and more uniform primary cell wall than wheat grain (Comino et al., 2014). During bread processing, the number of water-extractable-arabinoxylans was reported to increase from rye flour to bread, likely because of hydrolysis of water-unextractable-arabinoxylans (Cyran & Dynkowska, 2014). Arabinoxylan hydrolysis during baking reduced the proportion of high-molecular-weight extractable arabinoxylans and increased the proportion of low-molecular-weight arabinoxylans, as shown by Andersson et al., (2009). Overall, fiber solubilization, and the resulting increase in cell wall porosity may explain the increase in starch digestibility in bread compared to the flours but not the differences in starch digestibility in SB compared to MB and LB. The differences in SDS and RS content of the three bread samples could be mainly ascribed to the distinct texture of the bread. In general, rye proteins are not able to form a three-dimensional structure and a stable viscoelastic network, capable of holding gas during the fermentation as in wheat. Rye proteins are less prone to form intermolecular disulphide bonds and the presence of pentosan hinders the formation of a strong network (Beck et al., 2011). Hence, in rye bread, where the structure is mainly formed by a continuous phase of fiber, mainly arabinoxylans, connected with proteins that surrounded the starch granules, the use of a large flour particle size (> 1000 μm) decreased drastically the overall bread quality (Döring et al., 2015). Indeed, MB and LB showed a statistically higher

hardness than SB. Moreover, the crumb resilience and cohesiveness, which represent the ability of the crumb to regain its height after stress, decreased drastically with the increase in particle size. As suggested by Verbauwhede et al., (2018) and Renzetti et al., (2021) crumb cohesiveness and resilience in wheat bread are mainly controlled by starch swelling and amylose leaching rather than by the gluten network. This is also confirmed by Bressiani et al., (2017) who reported an increase in viscosity for flour with finer particle size than the coarse one, due to the large contact surface and the consequent starch leaching and swelling. This could explain why SB, produced with fine flour was more resilient and cohesive than MB and LB which contain large clusters of encapsulated starch in intact cells. Therefore, the different textural parameters reported for the three bread samples mirrored the different disintegration behaviors observed during the *in vitro* digestion. Indeed, MB and LB, which were characterized by a lower cohesiveness and resilience compared to SB, presented more than 50% of particles smaller than 0.3 mm^2 after the first 20 minutes of intestinal digestion. At the same time point, 70% of SB digesta particles were larger than 0.9 mm^2. The relatively bigger size of the compact digesta particles in SB could have limited the diffusivity of the enzymes inside the bread structure, slowing down starch digestibility, and therefore produced a higher amount of starch that escaped digestion, i.e., RS. Bread with the highest firmness and low moisture was the easiest to disintegrate during digestion and was not able to maintain its structure with the addition of digestive liquid (Bornhorst & Singh, 2012). The central role of disintegration rate in modulating starch digestibility was well explored *in vivo* by Vanhatalo et al., (2022). In the quoted manuscript, the disintegration rate of durum wheat food products with different textures, such as bread, cous cous and pasta, was evaluated and it was demonstrated that the food products with a more cohesive structure disintegrated less during the gastric phase, leading to bigger digesta particles, and therefore, to a reduced glycemic index. Hence, a highly packed and brittle structure

formed during bread processing is more easily weakened by intestinal movement during digestion. As shown in the manuscript, this facilitated the disintegration of the digesta into small particles and the consequent starch hydrolysis due to the greater contact surface between the enzyme and the substrate. The key role of crumb structure in modulating the starch digestibility of rye bread was also confirmed by Nordlund and co-workers (2016), who showed that, by increasing the porosity of the crumb structure through the addition of gluten in the bread recipe, the digestibility significantly increased. The formed porous aerated structure, indeed, was easier to disintegrate during digestion, and therefore, increased its insulin and glycemic response. From what was observed, it is possible to conclude that the driving mechanism in reducing starch digestibility in these rye model bread samples, where the structure is mainly formed by starch interaction and not gluten network, is the physical hindrance that enzymes face in reaching the starch. In support of this, also in starch gel, a simple food model, digestibility mainly depends on the texture and the mechanical properties of the matrix and, therefore, on the capacity of the enzyme to move into the gel. As Santamaria et al., (2021, 2022) reported, gels characterized by strong and highly structured architecture, including high firmness and a high elastic modulus (G'), show a lower starch digestibility than gel characterized by a porous and less organized structure with more viscous behavior (higher tan δ (G''/G')). This loose structure may facilitate easier enzyme penetration, thereby promoting the initial hydrolysis of starch. In conclusion, producing rye bread with flour of different particle sizes allowed to better understand the effect of intact cell walls on *in vitro* starch digestion in rye flour and bread. The intactness of cell walls is a limiting factor that controls the extent of hydrolysis of starch only in rye flour but not in a bread matrix. Instead, bread that disintegrated less during digestion was the one with the lower starch accessibility, confirming the central role of bread texture in modulating starch digestibility. The findings of the present study can guide future

experiments in enhancing crumb cohesiveness to reduce the disintegration during *in vitro* digestion and consequently lower starch digestibility. Moreover, in the future, it is advised to incorporate real mastication as a first step in the *in vitro* model of intestinal digestion. In such a way, a more realistic bolus disintegration can be achieved compared to the simplified sample preparation of the existing *in vitro* models of digestion. Even better, an acute intervention with bread with different textural properties where bolus properties are also characterized, should be carried out to confirm these speculations.

The effects of gluten addition, dough moisture content and mixing time on the textural properties and *in vitro* starch digestibility of durum wheat bread made with coarse semolina

Marianna Tagliasco, Donatella Peressini, Nicoletta Pellegrini

(In preparation)

Abstract

A promising approach to limit the starch accessibility to α-amylase in cereals is to preserve the integrity of the cell where the starch is encapsulated. However, this protective effect is lost when coarse semolina, rich in clusters of intact cells, is used to produce bread. It was hypothesized that the extended mixing time needed for optimal dough development increases cell wall porosity. Additionally, using coarse semolina compromises the bread crumb cohesiveness, accelerating its disintegration during digestion and thereby increasing starch accessibility. This study, therefore, aimed to explore the impact of dough mixing time (3.5 or 45 min), water absorption level (70%, optimum or 55%, low), and the use of 100% coarse semolina (S, particle size > 1000 µm) or its 20% substitution with vital gluten on textural properties (TPA) and *in vitro* digestibility (Englyst's method) of durum wheat bread. Bread prepared with 100% semolina at low hydration levels was the hardest, had the lowest volume, and had less rapidly digestible starch (RDS) compared to samples made with 20% gluten substitution and longer mixing times, which exhibited higher volume and porosity. These results indicated that, during the first 20 minutes of intestinal digestion, starch digestibility was predominantly influenced by crumb porosity, leading to more accessible starch in bread with a more aerated crumb structure. This was supported by a significant correlation (r= 0.82) between bread volume and RDS. However, in the subsequent 100 minutes of *in vitro* digestion, samples enriched with gluten, characterized by lower hardness and greater cohesiveness, displayed reduced slowly digestible starch, which was indeed strongly negatively correlated with cohesiveness (r=-0.90) and positively with hardness (r=0.88). This lower digestibility is likely due to better structural preservation during digestion, limiting crumb disintegration and starch accessibility. Thus, these findings suggest that replacing 20% coarse semolina with gluten could be an effective

strategy to decrease overall starch digestibility while maintaining desirable textural qualities in bread.

Keywords:

Coarse semolina; gluten; water absorption; mixing time; textural characteristics; *in vitro* digestibility.

4.1 Introduction

In Western countries, white wheat bread is daily consumed due to its affordability, palatability, and ease of incorporation into meals and, for this reason, the average consumption per capita stands at approximately 70 kg per year (De Boni et al., 2019). However, white wheat bread is characterized by a high GI (Atkinson et al., 2008; Scazzina et al., 2016), due to the complete starch gelatinization and a porous structure that facilitates contact between amylolytic enzymes and starch and speeds up the hydrolysis and subsequent glucose absorption. The overconsumption of highly digestible, highly glycemic starchy foods like bread is one of the contributors to the development of non-communicable diseases such as cardiovascular diseases and T2D (Chatterjee et al., 2017). Therefore, decreasing the starch digestibility of bread and in turn, its glucose response, has been the focus of several studies in the last decades. One possible approach to decrease starch digestibility is to limit the starch accessibility to α-amylase empowering the physical barriers present in the food matrix such as the one naturally present in flour, i.e., cell walls (Bhattarai et al., 2018), or formed during the process, such as the protein network (Li, Liet al., 2021). In cereals, the starch is naturally encapsulated in cells. However, during the milling process, the cells are intensively broken, and, therefore, in flour, starch becomes easily accessible to digestive enzymes (Tagliasco et al., 2022). On the contrary, the production of coarse flour with large particle size enables preservation of cell integrity resulting in flour containing clusters of intact cells. The starch digestibility of coarse wheat flour and minimally processed food, such as porridge, made with large particle size was significantly lower than the counterpart produced with fine particle size, both *in vitro* and *in vivo* (Edwards et al., 2015a; Korompokis et al., 2019; Tagliasco et al. 2022). However, when coarse flour with intact cells is employed to produce more processed food, such as bread, this protective effect seems to vanish. Korompokis et al. (2021) and

Tagliasco et al. (2022) found that, after the baking process, intact cells were still detectable in the product through microscopical analysis (e.g., CLSM), however, the rate of starch digestibility of bread made with coarse flour did not significantly differ from that of bread made with fine flour. During prolonged kneading (up to 90 minutes for the flour particles >1800 μm) and the fermentation step, the porosity of cell walls probably increased enhancing amylase accessibility to starch granules (Dornez et al., 2008). It was also hypothesized that incorporating coarse flour might have impeded the development of a dense gluten network, resulting in a significant reduction in crumb cohesiveness (Zhou et al., 2021). During digestion, a low bread crumb cohesiveness could potentially lead to an increase in the disintegration rate of the digesta, a decrease in particle size, and consequently, an increase in the rate of starch digestibility.

Besides the cell wall, the second barrier to starch digestibility in bread could be the gluten network formed thanks to flour hydration and the mechanical force applied during dough kneading (Li, Li et al., 2021). It has been demonstrated that the increased gluten density can act as a barrier between starch and digestive enzymes and decrease the extent of starch digestion (Xu et al., 2021; Zou et al., 2015). Additionally, the incorporation of gluten into dough not only physically hinders the contact between starch and enzymes, but can also bind pancreatic α-amylase, thereby inhibiting starch digestibility (Chen et al., 2019; López-Barón et al., 2017). Furthermore, incorporating gluten into the dough can also change the textural characteristics of bread by decreasing the hardness and chewiness and increasing the springiness and cohesiveness (Zeng et al., 2023). This can hypothetically modulate the disintegration of bread during digestion and consequentially the accessibility of enzymes to starch during intestinal digestion. Also, the modulation in dough hydration can modify bread texture, leading to products with denser structures which can lead to a reduced disintegration rate compared to traditional bread, and, consequentially, a lower starch digestibility (De La Hera et al., 2014;

Lau et al., 2015; Martinez et al., 2018). Several decades ago, it was hypothesized that lower starch digestibility in pumpernickel, a traditional rye bread, compared to common wheat bread was due to its compact structure limits its disintegration during digestion, leading to bigger particles of digesta that are more resistant to enzymatic diffusivity (Jenkins et al., 1986). Therefore, this study aimed to elucidate the effects of dough mixing time and hydration and gluten addition on bread texture and starch digestibility of durum wheat bread made with coarse semolina. It was hypothesized that by decreasing the mixing time, the porosity of the cell walls would not be increased retaining their barrier effect to starch digestibility. The addition of gluten could have a double effect in enlarging the density of the gluten network which entraps the starch granules and increases the cohesiveness limiting the disintegration rate during digestion. The ultimate aim of the present study is to understand how to produce bread with coarse semolina having acceptable textural properties and low *in vitro* starch digestibility.

4.2 Materials and methods

4.2.1 Materials

Peeled durum wheat was obtained from Duru BakliyatTM (Hediklik Diş Buğdayı, Turkey). Vital gluten from common wheat (*Triticum aestivum*) was obtained from Primeal (Peaugres, France). The total starch assay kit was purchased from Megazyme (Megazyme International, Ireland). The enzyme used to mimic gastrointestinal digestion were pepsin P7000 (from porcine gastric mucosa, specific activity ≥250 units/mg solid); pancreatin P7545 (from porcine pancreas, 8 × USP); invertase I4504 (from baker yeast, specific activity ≥300 units/mg solid); and amyloglucosidase A7095 (from *Aspergillus niger*, ≥260 U/mL) (Sigma Aldrich, Milan, Italy). All chemicals and solvents used were of analytical grade.

4.2.2 Methods

4.2.2.1 Coarse semolina production

Durum wheat was milled at room temperature by Hosokawa Micron, Laboratory Alpine® multi-processing system (Augsburg, Germany). Coarse semolina (S, particle size > 1000 µm) was produced using a fine impact mode (UPZ) and the following working conditions: 1550 rpm rotating speed, 1000 W power, 0.1 Nm^3/h UPZ bear, 60 m^3/h airflow, 360 Pa mill internal pressure, 280 Pa filter internal pressure and 3000 Pa filter differential pressure. The ground product was sieved and only the fraction of particles greater than 1000 µm was use for the experiments.

4.2.2.2 Dough preparation

Six different doughs were prepared using semolina or 20% vital gluten in substitution of S, 70% water absorption or 55% water absorption, and mixing times of 3.5 minutes or 45 minutes (Table 4.1). Doughs were prepared by mixing S or 20% vital gluten in substitution (80 g) with water at 63 rpm in a farinograph bowl at 30 °C (Promylograph T6, Max Egger, Austria). Water absorption 70% and 45 minutes corresponds to optimum water absorption (WA) and mixing time for dough made with 100% of S, respectively. The first was the amount of water (%) to be added to S (on 14% moisture basis) to reach a dough consistency of 500 BU (or 400 BU for 80 g S). The preparation of samples 80s20g required a two-step procedure: first the mixing of semolina and water for the time (t_s, min) required to bring the farinograph curve to a maximum, then the gluten was added and mixed until it reached a constant consistency (t_G, min). After mixing, the dough was kept for rheological measurement.

Table 4.1. Ingredients and farinograph mixing times used to prepare different dough samples.

	Semolina (%)	**Gluten (%)[a]**	**WA (%)[b]**	**Mixing time (min)[c]**
100s_45min_70%	100	-	70	45
100s_45min_55%	100	-	55	45
80s20g_45min_70%	80	20	70	41.5 + 3.5
80s20g_45min_55%	80	20	55	41.5 + 3.5
80s20g_3.5min_70%	80	20	70	3.5
80s20g_3.5min_70%	80	20	55	3.5

S: coarse semolina. G: gluten.

[a] $G = (G \times 100) / (CS + G)$

[b] on semolina or S-G blend basis

[c] The double values indicate $t_S + t_G$

4.2.2.3 Dough rheological properties

The extensional properties of doughs at 25 °C were measured using a TA-XT Plus Unit Texture Analyzer (Stable Micro Systems, Godalming, UK), which was equipped with a 5 kg load cell and a Kieffer dough/gluten extensibility rig. After mixing (see 2.2.2), the dough (~50 g) was immediately transferred into a humidity-controlled chamber (80% relative humidity) and rested for 20 minutes to relax. Then, it was placed in a lubricated Teflon mold and compressed for 40 minutes to be shaped in 5 cm long strips with a trapezium cross-section. Finally, the tension in the clamp was released and the dough rested for 10 minutes in the mold. Before testing, both ends of the strip sample was clamped between the plates of the Kieffer rig. Then, the dough was subjected to a uniaxial extension at 3.3 mm/s until fracture. From the force vs. displacement curve, the peak force or fracture force and the displacement at peak force were taken as a measure of maximum resistance to extension (Rmax, N) and extensibility (Ep, mm), respectively. For each dough, twelve strips were analyzed in duplicate.

4.2.2.4 Breadmaking

A standard recipe was used to produce bread (Tagliasco et al., 2022), which included semolina or semolina-gluten blend (80 g), salt (0.8 g), dry yeast (0.96 g) and water (Table 4.1). Doughs were mixed in the farinograph bowl for 3.5 or 45 minutes, as described in paragraph 4.2.2.2 (Promylograph T6, MaxEgger, Austria). After mixing, the dough was transferred to a plastic bowl and kept for 52 minutes at 30 °C and 80% relative humidity for the first step of fermentation. Then, the dough was pounced for 30 s to redistribute the gas bubble and molded in a baguette shape, placed in a greased aluminum pan (10 ×7 × 4 cm) and fermented for 50 minutes (30 °C and 80% relative humidity). The proven dough was baked at 200 °C for 20 minutes in a professional oven (Electrolux, model A0S101ETA1, Stockholm, Sweden). After baking, bread was removed from the aluminum pan and left cooling at ambient temperature (~21 °C) for 1 hour. For further analyses, samples were packaged in Ziploc® quart freezer plastic bags 17.7 x 18.8 cm (SC Johnson, USA) and analyzed the day after.

4.2.2.5 Bread chemical composition

The moisture content of bread crumb was determined according to the AACC Approved Method 44-15.02. (AACC, 1999). The total starch was evaluated using the assay kit provided by Megazyme (Bray, Ireland) following the procedure: Determination of total starch content of samples containing resistant starch (RTS-NaOH Procedure - Recommended). For the analysis, bread crumb was dried in a vacuum oven (Vuotomatic 50, Bicasa, Milan, Italy) at 70°C for 16 h, then milled to obtain a powder with a particle size below 500 mm, as suggested by the methodology. Protein content (N x 5.7) was measured using the Kjeldahl method (Kirk, 1950).

4.2.2.6 Bread volume

Bread volume (cm^3) was measured by the rapeseed displacement method according to the AACC Approved Method 10-05.01 (AACC, 2009). Determinations were performed in triplicate.

4.2.2.7 Textural characterisation

The textural properties of bread were evaluated by performing a texture profile analysis (TPA) using a TA-XT plus analyzer equipped with a load cell of 30 kg. (Stable Micro Systems, Godalming, UK). Bread was cut into slices (25 mm height) and the central portion of the crumb was taken using a cylindric mold of 20 mm diameter. The samples were subjected to double compression using a cylindric probe of 36.0 mm diameter (P/36). The setting of the test was: 1.00 mm/s speed, 40% compression and a resting time of 5 s between the first and second compression. The following textural properties were obtained from the force vs. time curve: hardness (peak force of the first compression, N), springiness (ratio between the time duration of force input during the second compression and that obtained during the first compression, dimensionless), cohesiveness (ratio of the area under the second cycle to the area under the first cycle, dimensionless). For each type of bread, 12 bread slices were analyzed from 3 batches.

4.2.2.8 Image analysis of breadcrumb

Representative images of bread slices were acquired using a digital camera Canon reflex EOS 550D (Canon Inc., Tokyo, Japan) with an EF-S 60 mm f/2.8Macro USM lens. The distance between bread and the camera lens was fixed at 48 cm and the light was controlled. Crumb porosity, expressed as % representing the percentage of pores in each bread slice, and the number of pores were calculated using ImageJ (version 1.52a, National Institute of Health). The taken images were converted in 8-bit, modified for brightness/contrast, employing a threshold around 0-100. In the data analysis, particles smaller than

0.30 mm^2 were excluded for each image to avoid interference with the background. Two slices for each bread production were processed and bread was produced three times in three days.

4.2.2.9 *In vitro* starch digestibility

The starch digestibility of bread samples was assessed following the methodology described by Englyst et al. (2018) and in Tagliasco et al. (2022). The test was performed on around 2 g of breadcrumbs previously cut into small pieces (5 × 5 ×5 mm^3). The *in vitro* starch digestibility test consists of two parts, the first one, lasting 30 minutes, mimics the gastric phase and the second one, lasting 120 minutes, reproduces the intestinal digestion. During the first phase, 10 mL of pepsin-guar solution (0.05 M HCl) was added to bread and the digesta was incubated for 30 minutes in a water shaking bath (37 °C, 180 rpm (3Hz)). Then, in the digesta were added 10 mL of 0.25 M sodium acetate buffer (37 °C), 5 marbles and 5 mL of enzyme mixture containing: pancreatin (8 USP), amyloglucosidase (≥ 260 U/mL) and invertase (≥ 300 units/mg solid). The samples were shaken in the water bath for 120 minutes at 37°C and 180 rpm. The digesta were sampled twice during the intestinal phase: at 20 minutes (T20) and 120 minutes (T120). The enzymatic reaction was stopped using ethanol at 96% (vol/vol) and the glucose produced during digestion was detected using GOPOD reagent (assay kit from Megazyme Bray, Ireland). The absorbance was measured at 510 nm using a spectrophotometer (Agilent Technologies, USA). Rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) were the principal data obtained from Englyst's digestion (Englyst et al., 1996). RDS was calculated by multiplying the amount of glucose released in the first 20 minutes of intestinal digestion by 0.9 to get the amount of starch. SDS represented the starch digested between T20 and T120 minutes. RS corresponds to the not-digested starch, so the differences between the total starch and the amount of starch digested after 120 minutes. RDS, SDS and RS were expressed as percentages of digested starch on the

total starch (wet basis). The starch digestibility was evaluated three times in triplicates (n=9).

4.2.3 Statistical analysis

The results were reported as mean ± standard deviation and analyzed with the software IBM SPSS Statistics 25 (IBM, Armon, USA). Multivariate analysis of variance (MANOVA) was used to evaluate the effects of mixing time, hydration, gluten addition and their interaction on the textural properties and starch digestibility of durum wheat bread made with coarse semolina ($\alpha = 0.05$). The percentage of the total variation was computed to explain the variance of each parameter as a function of the sum squares of the main factors and their interaction. Moreover, a post hoc Tukey test ($p < 0.05$) was used to determine which samples differed from the others. A principal component analysis (PCA) was performed using the correlation matrix. The Pearson correlation coefficients among the parameters were calculated, and their significance was tested at a significance level of 0.05.

4.3 Results

4.3.1 Dough mixing properties

Fig. 4.1 shows farinograms (consistency vs. mixing time) for doughs at two different absorption levels. Dough prepared exclusively with semolina exhibited an optimum WA value of 70%. For dough made with only semolina particles started to aggregate after a mixing time of 5 minutes, and an additional 40 minutes are required to obtain a dough with maximum consistency, which is related to a developed gluten. As expected, maximum consistency was greater for the dough at 55% WA (Fig. 4.1a, b). Looking at dough mixing behavior, it was not feasible to produce a dough with 100% semolina by mixing for a short time. As reported previously, it took more than 5 minutes of kneading for hydrated semolina particles to form protein fibrils on their surface and stick together, and 45 minutes for dough development due to coarse semolina granulation. The addition of vital gluten gave a transition from hydrated semolina aggregates into a dough in a short time of mixing (3.5 min) due to its fast water absorption and development of a gluten network (Fig. 4.1e, f). Therefore, 80s20g_3.5min samples are relevant. For 80s20g_45min doughs, a two-step process was applied: a) semolina and water were mixed to develop gluten from durum wheat; b) vital gluten was added to the dough and mixed until a peak consistency was reached contributing to dough microstructure (Figs. 4.1c, d).

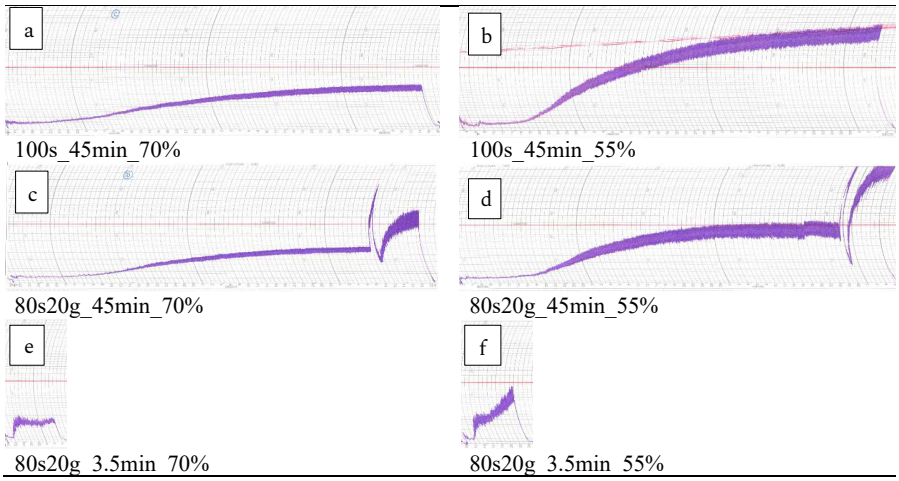


Figure 4.1. Farinograph curves of doughs made with 100% semolina (100s), partly substituted with 20% gluten (80s20g), mixing time of 3.5 minutes or 45 minutes and different hydration levels (55% or 70%). S = semolina; g = gluten.

4.3.2 Dough rheological properties

Elongation tests were carried out to investigate dough viscoelastic properties, which are recognized as relevant determinants of bread quality (Peressini et al., 2017). Tables 4.2 and 4.3 shows dough extensional properties and results of multivariate analysis of variance to understand the effects of different parameters (gluten addition, hydration level and mixing time) on dough and bread properties. Large differences in maximum resistance to extension (Rmax) and extensibility at fracture (Ep) were observed between different doughs. The addition of 20% gluten significantly increased Rmax and Ep ($p \leq 0.01$) (Table 4.3). Rmax was the highest for the dough 80s20g_45min_55%, and the lowest for the 100s_45min_70% dough sample. Moreover, Ep was the highest for 80s20g_3.5min_70% dough sample and the lowest for 100s_45min_55%. Hydration also significantly affected the Rmax ($p \leq 0.01$) and Ep ($p \leq 0.001$) (Table 4.3). Comparing the samples made with the same recipe and mixing time, the dough with higher hydration exhibited lower Rmax than the

doughs produced with 55% WA indicating a softening effect of water. Conversely, the extensibility increased with the increase in WA. The mixing time had a lower but significant effect compared to the other variables on Rmax ($p \leq 0.05$) and Ep ($p \leq 0.05$). To summarize, gluten addition increased resistance to extension and extensibility improving dough viscoelastic properties due to the development of an adequate protein network. Furthermore, the addition of water reduced resistance and improved extensibility of the dough.

Table 4.2. Maximum resistance to extension (Rmax) and the extensibility (Ep) of dough made with 100% coarse semolina (100s), partly substituted with 20% gluten (80s20g), mixing time of 3.5 minutes or 45 minutes and different hydration levels (55% or 70%).

	Rmax (N)	**Ep (mm)**
100s_45min_70%	0.17 ± 0.01^{e}	11.00 ± 0.03^{cd}
100s_45min_55%	0.36 ± 0.02^{d}	6.59 ± 1.15^{d}
80s20g_45min_70%	0.94 ± 0.13^{b}	20.80 ± 2.32^{b}
80s20g_45min_55%	1.67 ± 0.31^{a}	12.30 ± 0.3^{cd}
80s20g_3.5min_70%	0.45 ± 0.05^{c}	29.37 ± 5.00^{a}
80s20g_3.5min_55%	0.93 ± 0.20^{b}	15.97 ± 1.83^{bc}

Values are expressed as mean ± SD. Mean values (n=9) within a column with different letters were significantly different ($p< 0.05$; Tukey's test). s = semolina; g = gluten.

Table 4.3. Multivariate Analysis of Variance (MANOVA) based on Pillai's Trace test of the effect of three fixed variables: gluten (G), hydration (H_2O%) and mixing time (MT) on the characteristics of dough and bread samples.

		Rmax	Ep	Volume	HRD	SPR	CHS	N° pores	Porosity	RDS	SDS	RS
G	SS%	63.5	29.2	63.5	92.7	100	91.8	56.0	68.2	61.6	91.7	73.6
	P	***	**	***	***	***	***	***	*	**	*	ns
H_2O%	SS%	14.4	52.6	1.7	0.4	0	4.3	5.75	15.2	15.9	6.9	0.4
	P	**	***	ns	ns	ns	ns	*	ns	*	ns	ns
MT	SS%	22.1	18.2	34.7	6.9	0	4.4	38.2	16.6	22.5	1.5	25.9
	p	*	*	***	ns	ns	ns	ns	ns	*	ns	ns

SS, sum of squares; P, significance; *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ns: not significant. Dough maximum resistance to extension (Rmax), dough extensibility (Ep), bread volume; bread hardness (HRD); bread springiness (SPR); bread cohesiveness (CHS); rapidly digestible starch (RDS); slowly digestible starch (SDS); resistant starch (RS).

4.3.3 Bread characterization

The proximate composition of bread loaves is displayed in Table 4.4. The three bread samples produced with 70% hydration had similar moisture content which was significantly higher than the ones of the three bread samples made with 55% hydration. The total starch of bread 100s_45min_55% was the highest followed by 100s_45min_70%, not significantly different from 80s20g_45min_55% and 80s20g_3.5min_55%. Bread 80s20g_45min_70%, characterized by higher moisture content and 20% gluten addition, presented the lowest amount of total starch. The level of protein in bread samples was significantly lower for bread loaves made with 100% semolina than bread samples prepared with the substitution of 20% gluten.

Table 4.4. Moisture content, total starch and protein content of bread made with 100% coarse semolina (100s), partly substituted with 20% gluten (80s20g), mixing time of 3.5 minutes or 45 minutes and different hydration levels (55% or 70%).

	Moisture content (%)	**Total starch (g/100 g fresh bread)**	**Protein content (g/100 g fresh bread)**
100s_45min_70%	44.31 ± 0.70^{a}	34.71 ± 0.01^{b}	8.62 ± 0.9^{c}
100s_45min_55%	37.94 ± 0.91^{b}	39.10 ± 0.02$_{a}$	9.78 ± 0.1^{c}
80s20g_45min_70%	43.82 ± 0.60^{a}	29.15± 0.02^{c}	15.1 ± 0.3^{b}
80s20g_45min_55%	36.80 ± 0.84^{b}	33.84± 0.00^{b}	17.34 ± 1.1^{ab}
80s20g_3.5min_70%	43.32 ± 1.66^{a}	29.61± 0.01^{c}	16.77 ± 0.6^{ab}
80s20g_3.5min_55%	38.01 ± 0.56^{b}	33.6 ± 0.03^{b}	16.6 ± 0.7^{ab}

Values are expressed as mean ± SD. Mean values (n=9) within a column with different letters were significantly different ($p < 0.05$; Tukey's test).
s = semolina; g = gluten.

4.3.4 Textural properties of bread

The textural properties of the six samples were evaluated to study the influence of the variables, such as gluten addition, hydration and mixing time, on the overall quality of bread samples (Table 4.5). The volume was significantly affected by both gluten addition ($p \leq 0.001$) and mixing time ($p \leq 0.001$), but not by hydration (Table 4.3). Among the six breads, the two samples prepared with 20% gluten and optimum mixing time (45 min) showed the largest volume, despite the different hydration levels. As regards the effect of mixing time on volume, bread 80s20g_3.5min_55% and 80s20g_3.5min_70% showed a volume significantly lower than the ones with optimum mixing times (45 min). Interestingly, bread 80s20g_3.5min_70%, regardless of the addition of gluten, showed the lowest volume along with bread samples made with 100% semolina. Among the textural

characteristics, hardness was significantly correlated with bread volume (r=-0.85) (Table 4.7). As shown in Table 4.5, bread with a larger volume exhibited a softer structure than samples with lower volume, which were characterized by a harder crumb. Hardness was mainly affected by gluten addition ($p \leq 0.001$), as shown in Table 4.3. Indeed, samples with gluten addition were notably softer than bread made solely with semolina. The only sample containing gluten with a significantly higher hardness was 80s20g_3.5min_70%. Among the six samples, bread made with 100% semolina and low hydration was the hardest. Cohesiveness and springiness were significantly affected by the gluten addition ($p \leq 0.001$) (Table 4.3), and they were significantly higher in bread made 20% gluten substitution. Instead, bread samples produced with 100% semolina showed the lowest value of cohesiveness.

Table 4.5. Volume, hardness, cohesiveness, springiness of bread made with 100% coarse semolina (100s), partly substituted with 20% gluten (80s20g), mixing time of 3.5 minutes or 45 minutes and different hydration level (55% or 70%).

	Volume (cm^3)	Hardness (N)	Cohesiveness (-)	Springiness (-)
100s_45min_70%	111.7 ± 12.6^{c}	23.8 ± 0.0^{b}	0.71 ± 0.01^{c}	0.95 ± 0.00^{ab}
100s_45min_55%	90.0 ± 13.2^{c}	43.8 ± 10.5^{a}	0.70 ± 0.01^{c}	0.92 ± 0.00^{b}
80s20g_45min_70%	315 ± 13.2^{a}	3.8 ± 0.4^{c}	0.82 ± 0.01^{a}	0.97 ± 0.00^{a}
80s20g_45min_55%	286.7 ± 31.7^{a}	4.0 ± 3.2^{c}	0.81 ± 0.02^{a}	0.97 ± 0.00^{a}
80s20g_3.5min_70%	105.0 ± 5.0^{c}	22.3 ± 3.2^{b}	0.77 ± 0.01^{b}	0.94 ± 0.02^{b}
80s20g_3.5min_55%	205.0 ± 8.7^{b}	4.5 ± 0.7^{c}	0.81 ± 0.01^{a}	0.97 ± 0.0^{a}

Values are expressed as mean ± SD. Mean values (n=9) within a column with different letters were significantly different ($p < 0.05$; Tukey's test). s =semolina; g=gluten.

4.3.5 Porosity and bread structure

The representative images of bread slides are shown in Figure 4.2 and the results from image analysis of breadcrumbs are presented in Table 4.6. The gluten addition significantly affected the number of pores ($p \leq 0.001$) and porosity ($p \leq 0.05$) (Table 4.3) and among the analyzed samples, all bread samples with gluten addition, apart from 80s20g_45min_70%, exhibited the highest number of pores and the greatest porosity percentage. The sample 100s_45min_70% presented a number of pores significantly higher than 100s_45min_55% and 80s20g_45min_70% due to the gluten network formed by endogenous gluten network developed during kneading for the optimum mixing time at the appropriate hydration level. On the other hand, bread 100s_45min_55% and 80s20g_45min_70% exhibited the lowest number of pores and the least porosity among all bread samples. Representative images of the entire slices displayed the difference in structure among the six bread samples. The bread characterized by high porosity showed a higher cross-sectional height than the bread characterized by lower porosity and number of pores. The crumb of bread samples produced with 45 minutes of mixing exhibited a uniform homogeneous crumb, instead in the two bread samples mixed for 3.5 minutes, the semolina particles were still visible, and not well embedded in the bread structure.

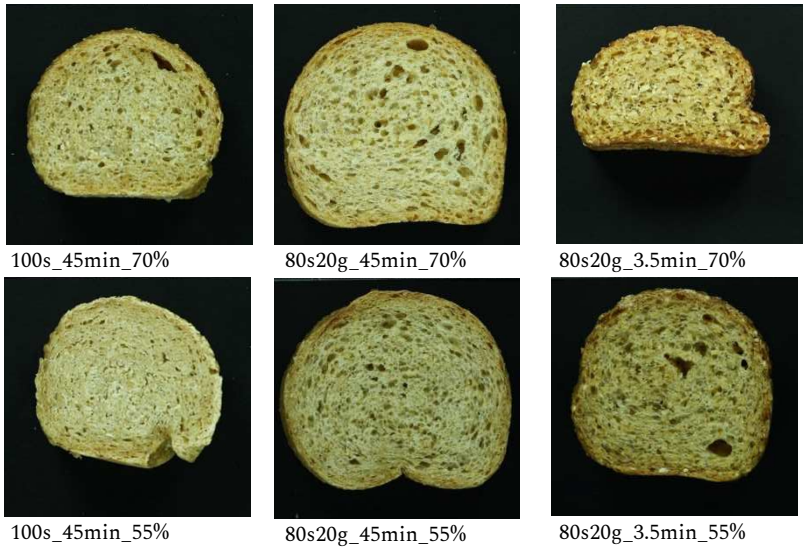


Figure 4.2. Representative images of bread made with 100% coarse semolina (100s), partly substituted with 20% gluten (80s20g), mixing time of 3.5 minutes or 45 minutes and different hydration levels (55% or 70%).

Table 4.6. Number of pores and porosity (%) of bread made with made with 100% coarse semolina (100s), partly substituted with 20% gluten (80s20g), mixing time of 3.5 minutes or 45 minutes and different hydration levels (55% or 70%).

	N° of pores	**Porosity (%)**
100s_45min_70%	207.3 ± 19.4^{b}	15.6 ± 2.8^{ab}
100s_45min_55%	143.5 ± 31.1^{c}	7.1 ± 1.7^{b}
80s20g_45min_70%	351.3 ± 26.3^{a}	16.7 ± 2.1^{a}
80s20g_45min_55%	293.7 ± 30.4^{a}	13.9 ± 3.4^{a}
80s20g_3.5min_70%	86.2 ± 46.2^{c}	10.3 ± 3.4^{b}
80s20g_3.5min_55%	294.5 ± 40.5^{a}	16.3 ± 2.4^{a}

Values are expressed as mean ± SD. Mean values (n=9) within a column with different letters were significantly different ($p< 0.05$; Tukey's test). s =semolina; g=gluten.

4.3.6 Starch digestibility

RDS was significantly affected by gluten addition ($p \leq 0.01$), hydration ($p \leq 0.05$) and mixing time ($p \leq 0.05$) (Table 4.3) and showed a strong correlation with volume (r= 0.82) (Table 7). Bread sample 100s_45min_55%, which had the smallest volume, had the lowest level of RDS, instead 80s20g_45min_70%, with the biggest volume, had the highest level of RDS. Among the other samples, no significant difference was detected. SDS was significantly affected by gluten ($p \leq 0.05$) (Table 4.3) and exhibited a significant decrease in bread samples produced with 20% gluten substitution compared to the samples made with 100% semolina. SDS also showed a strong correlation with hardness (r = 0.88) and an inverse correlation with cohesiveness (r = -0.90) (Table 4.7). Bread having higher hardness but lower cohesiveness, such as the samples made with 100% semolina, showed a higher SDS compared to the other samples. RS was not significantly different among the six bread samples and not affected by one of the studied parameters. However, it was slightly higher in bread samples with 20% gluten. The sum of RDS+SDS, which represents the amount of starch digested at the end of the *in vitro* digestion was significantly higher in samples made with 100% semolina than the bread made with 20% gluten substitution.

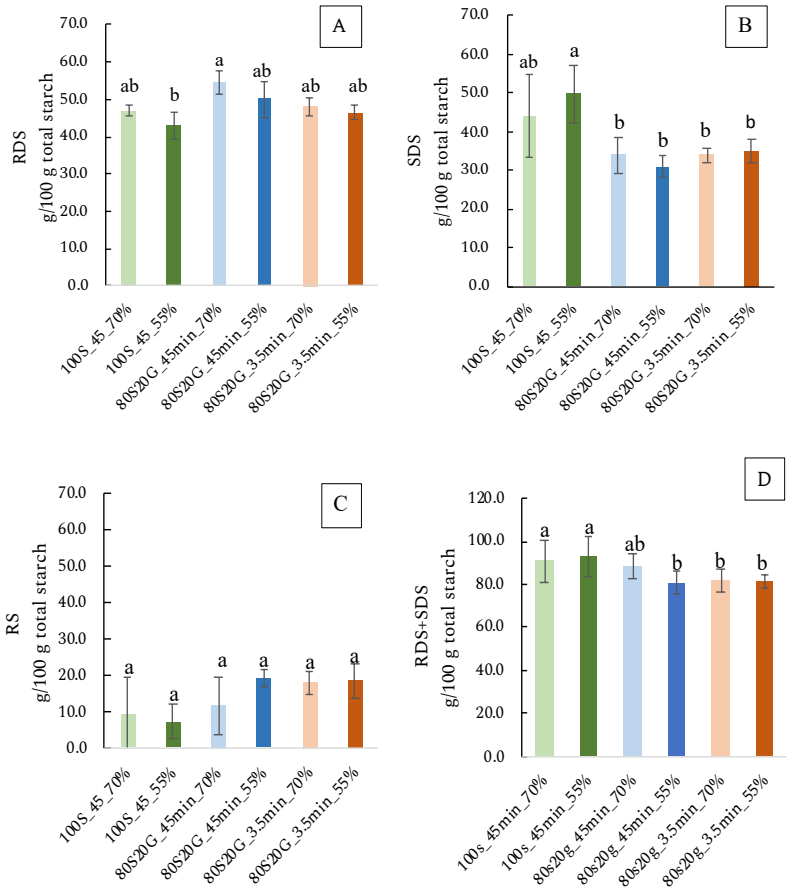


Figure 4.3. Rapidly digestible starch (RDS) (A), slowly digestible starch (SDS)(B), resistant starch (RS) (C) and RDS+ SDS (D) of bread samples made with 100% coarse semolina (100s), partly substituted with 20% gluten (80s20g), mixing time of 3.5 minutes or 45 minutes and different hydration levels (55% or 70%). Columns sharing the same letter were not significantly different ($p < 0.05$; Tukey's test) (n=9)

Table 4.7. Pearson's correlation matrix between dough and bread characteristics and starch digestion parameters.

	R max	**Ep**	**vol**	**HRD**	**CHS**	**SPR**	**Porosity**	**N° pores**	**RDS**	**SDS**	**RS**
Rmax	1										
Ep	0.00	1									
vol	0.84*	-0.1	1								
HRD	-0.74	0.35	-0.85*	1							
CHS	0.82*	-0.44	0.86*	-0.92**	1						
SPR	0.75	-0.13	0.91*	-0.96**	0.84*	1					
Porosity	0.350	-0.13	-0.73	-0.83*	0.58	0.90*	1				
N° pores	0.658	0.16	-0.91*	-0.79	0.70	0.91*	0.80	1			
RDS	0.51	-0.50	0.82*	-0.76	0.72	0.76	0.63	0.64	1		
SDS	-0.73	0.63	-0.71	0.88*	-0.90*	-0.75	-0.52	-0.46	-0.73	1	
RS	0.65	-0.52	0.38	-0.67	0.74	0.49	0.27	0.17	0.28	-0.86*	1

Dough maximum resistance to extension (Rmax); dough extensibility (Ep); bread volume (vol); bread hardness (HRD); bread springiness (SPR); bread cohesiveness (CHS); rapidly digestible starch (RDS); slowly digestible starch (SDS); resistant starch (RS).

*Correlation is significant at the 0.05 level.

**Correlation is significant at the 0.01 level.

4.3.7 Principal Component Analysis

The use of principal components analysis aims to analyze the whole set of data and attribute specific features to the sample bread (Figure 4.4a). The first component (PC1) accounting for 68.4% of the total variation was expressed as a function of dough Rmax and bread volume, hardness, cohesiveness, springiness, porosity, number of pores, and RDS, while the second component (PC2) accounting for 16.4% was expressed as a function of dough extensibility and bread digestibility in terms of SDS and RS. PC1 and PC2 together accounted for 84% of the total variance. Figure 4.4b shows bread samples projected into the factorial space and divided into two main clusters. In the right upper corner were placed bread 80s20g_3.5min_55%, 80s20g_45min_55% and 80s20g_3.5min_70% that were well correlated with preferable bread textural characteristics such as cohesiveness, springiness, porosity, and volume, and negatively correlated with hardness. For what concern digestibility, they were characterized by high RDS and RS and low SDS. In the opposite corner, were allocated bread samples produced with 100% coarse semolina despite the hydration level, and they were characterized by high SDS, and high hardness and were inversely correlated with volume, porosity, cohesiveness, RDS and RS.

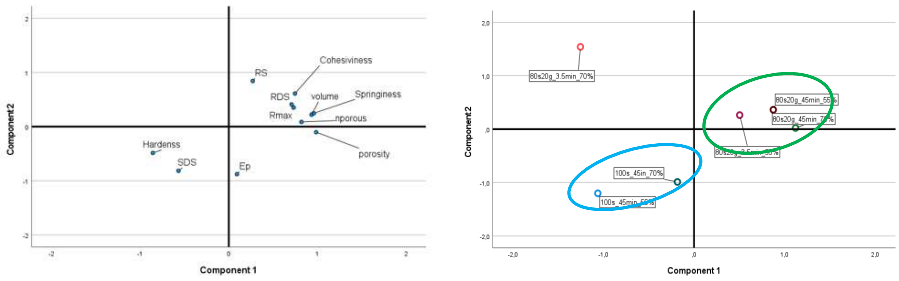


Figure 4.4. Principal components analysis of textural and nutritional characteristics of the six bread samples produced with coarse semolina, gluten addition and different hydration levels. (A): biplot of the first two components; (B): rotated principal scores of six bread samples produced. Maximum resistance to extension (Rmax) and extensibility (Ep) of dough; bread volume; bread hardness; bread springiness; bread cohesiveness; bread porosity percentage, bread number of pores, rapidly digestible starch (RDS); slowly digestible starch (SDS); resistant starch (RS).

4.4 Discussion

The reduction of blood glucose response to starchy food has been the focus of several investigations in the last decades. A promising strategy for limiting starch accessibility to α-amylase in cereals is the use of coarse flour where the starch is encapsulated in cells whose integrity is mostly retained (Bhattarai et al., 2018). However, using coarse flour for bread production requires prolonged mixing time, which probably increases cell wall porosity and enhances enzyme penetration. Moreover, coarse semolina bread is hard and has a reduced cohesiveness that increases the disintegration rate during digestion and, in turn, the starch accessibility (Korompokis et al., 2021; Tagliasco et al. (2022)). Thus, the present study investigated several approaches, i.e., semolina substitution with 20% gluten, short mixing time, and low dough hydration, in order to produce durum wheat bread made with coarse semolina with good textural properties and low *in vitro* starch digestibility.

Durum wheat is characterized by tenacious and inextensible gluten, which leads to doughs that are less extensible and difficult to handle (Sapirstein et al., 2007; Sissons, 2008). Moreover, the presence of large particles in dough made with 100% coarse semolina limited the development of the gluten network weakening the structure in agreement with previous studies (Bressiani et al., 2017; Korompokis et al., 2021). Indeed, 100% coarse semolina doughs had low resistance to extension and low extensibility, and bread samples were characterized by hard crumbs, reduced volume and a low level of cohesiveness and springiness. Moreover, the reduction in dough hydration in the bread samples significantly increased the hardness, as previously reported by Martínez et al. (2018) and De La Hera et al. (2014). Low hydration level, indeed, hindered the formation of the gluten network during kneading due to a competitive water absorption between starch and proteins. Such competition impedes the intermolecular interaction among ingredients and the formation of hydrogen bonds, the hydrophobic interaction and the sulfhydryl links that contribute to the complete formation of a developed gluten network (Lyu et al., 2023; Yang et al., 2021). Gluten addition, instead, generally improved the dough characteristics and the overall quality of bread, decreasing hardness and increasing volume due to the well-developed viscoelastic gluten. However, the gluten strength is influenced by the number of entanglements between glutenin chains, which depends on protein quantity and quality (Dobraszczyk & Salmanowicz, 2008; McCann & Day, 2013). A large number of proteins (in particular long chains glutenins) enhances extensibility since molecules can slide on each other without causing a coherence loss (Bloksma, 1990).

Besides the increased gluten content in the dough, the substitution of 20% semolina with vital gluten improved the textural quality thanks to its high functionality. Such gluten was from common wheat (*Triticum aestivum*) which contains a specific allele at the glutenin Glu-D1 locus that is absent in durum wheat (semolina). This allele encodes HMW glutenin (HMW-GS) which is fundamental in the formation of a proper

viscoelastic gluten network suitable for bread-making (Cecchini et al., 2021; Mastrangelo & Cattivelli, 2021). The gluten substitution also generally increases resistance to extension and improves extensibility in dough and this results in a large bread volume (Peressini et al., 2017). The substitution with vital gluten, moreover, made the crumb more elastic and therefore resistant to deformation increasing cohesiveness and springiness, textural properties that quantify the internal resistance and cohesion of food structure upon deformation. However, among bread samples made with 20% gluten substitution, the ones mixed for a short mixing time (3.5 min) showed a lower volume than the ones mixed for the optimum kneading time (45 min), despite the different hydration levels. The short mixing time did not allow the development of the endogenous gluten network of semolina, and the bread volume was primarily due to the added vital gluten. In addition, 80s20g_3.5min_70% bread showed a similar volume as bread samples made with 100% semolina. The high hydration and a short mixing time prevented complete water absorption and caused the collapse of the structure during the fermentation and the baking steps. To sum up, as shown by the PCA, the substitution of 20% semolina with vital gluten and the right amount of water related to the mixing time can significantly improve the textural features of bread made with coarse semolina.

Regarding the starch digestibility, we found that RDS, which represents the starch digested in the first 20 minutes of *in vitro* intestinal digestion, was highly correlated with volume (r= 0.82). Consequently, 100s_45min_55% bread, which was characterized by the smallest volume among bread samples, displayed the lowest RDS value. The reduced volume and the firm crumb (44 N), resulting from low hydration and the absence of gluten substitution, might have limited the physical breakdown during the first 20 minutes digestion leading to reduced digested starch (Martínez et al., 2018; De La Hera et al., 2014). As clearly shown in Chapter 3, bread with a hard crumb structure, like 100s_45min_55%, remained intact during the initial 20

minutes of *in vitro* intestinal digestion. Consequently, enzymes had limited accessibility to hydrolyze starch compared to the porous aerated structure of other samples. This finding aligns with Freitas et al. (2022), who compared starch digestibility of bread with similar composition but different densities and found that harder denser bread structures were less digestible than softer voluminous bread. Prolonging the intestinal digestion, other bread physical characteristics became prominent. Indeed, the quantity of starch digested between 20 minutes and the end of the simulated intestinal digestion (120 min) (i.e., SDS) was strongly correlated with hardness (r= 0.88) and inversely correlated with cohesiveness (r= -0.90). Consequentially, bread samples made with 100% semolina, which were characterized by higher hardness and lower cohesiveness compared to the other samples, exhibited higher SDS values. During the bread making, the use of only coarse semolina weakened the gluten network making it more susceptible to rupture and crumble upon deformation (Bressiani et al., 2017; Korompokis et al., 2021). In contrast, the substitution of 20% semolina with vital gluten enhanced bread cohesiveness, potentially reducing digesta disintegration and, consequently, enzyme accessibility during the last 100 minutes of digestion. Additionally, the 20% gluten substitution may have increased the density of the gluten network surrounding the starch granules (Xiong et al., 2023; Xu et al., 2021).

Furthermore, peptides formed during fermentation might bond with starch, limiting starch accessibility, or directly inhibiting the amylolytic enzymes (Liu et al., 2021; Lu et al., 2023; Xiong et al., 2023). These factors, collectively, led to an overall reduction in starch digestion at the end of *in vitro* experiments in bread samples incorporating 20% gluten, regardless of the degree of hydration and mixing time. In line with these results, Zeng et al. (2023) observed a decrease in starch digestibility by increasing the amount of gluten added to the bread recipe. However, they observed a different trend where RDS decreased and SDS increased by increasing the level of

gluten. In the quoted study, bread samples were produced with fine whole wheat flour and their digestion led to a value of RDS of around 79% (Zeng et al., 2023). Instead, in the present study, the RDS ranged around 50%. Probably, the starch encapsulated in intact cell walls, as reported in Tagliasco et al. (2022), slowed down the starch hydrolyzation, due to the physical hindrance between starch and enzymes. Therefore, it is possible to hypothesize that, in the present study, there was an overall delay in starch digestibility due to the use of coarse flour.

In conclusion, it is possible to produce durum wheat bread with coarse semolina with acceptable textural properties and low *in vitro* starch digestibility by replacing 20% flour with vital gluten. Besides, development of a strong gluten network to improve the bread quality may hamper the enzyme activity and lead to a cohesive crumb texture, which does not completely disintegrate during digestion reducing the enzyme diffusivity inside the bread structure. However, these results must be confirmed by a human study comprising the oral processing of bread, which is the first phase of human digestion and that significantly influences the disintegration rate of food.

5 Chapter

The combined effect of gluten addition and cell wall integrity in durum wheat bread on oral processing, postprandial glycemic and insulinemic responses in heathy volunteers

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(In preparation)

Abstract

Decreasing the blood glucose response of starchy food and consequentially its glycemic index has been the focus of several research in the last decades. One strategy used to reduce GI is to limit the starch accessibility to α-amylase preserving the integrity of the cell where the starch is encapsulated. However, the use of coarse flour, rich in clusters of intact cells, adversely affected bread texture, reducing crumb cohesiveness, increasing disintegration rate, and consequently enhancing starch accessibility. In our previous experiments, the substitution of 20% coarse semolina with gluten improved bread crumb quality, increasing cohesiveness and reducing hardness, while slightly decreasing *in vitro* starch digestibility. This study aimed to confirm in healthy volunteers the combined effect of coarse semolina and 20% gluten substitution on glycemic and insulinemic responses. Sixteen volunteers participated in tests, which were carried out following the International standards organization (ISO) guidelines, and randomly consumed bread made with coarse semolina and 20% gluten substitution (80CS_20G), its counterpart with fine semolina (80FS_20G), and a third sample made with fine semolina and 5% gluten substitution (95FS_5G). The oral processing of these bread samples was also evaluated to understand how gluten and semolina particle sizes affect oral disintegration and the release of reducing sugars during mastication. Among the three samples, no differences in glycemic responses and mastication behavior were observed. Bread 80CS_20G and 80FS_20G exhibited similar textural properties, but the former released a lower amount of reducing sugars in the bolus, leading to a reduced insulin response 30 minutes post-consumption than the latter. The intact cell clusters present in coarse semolina of bread (i.e., 80CS_20G) may have hindered and slowed starch accessibility during the oral and early gastric phases, thereby eliciting a lower insulin response. Also, 95FS_5G, after mastication released a

lower amount of reducing sugars and lower insulin production than 80FS_20G. The compact structure of 95FS_5G may have delayed starch hydrolysis at the oral level by restricting α-amylase accessibility compared to the more porous voluminous 80FS_20G, whose structure may have facilitated and speed up the access of salivary α-amylase to starch granules. These differences in insulin production could have potentially mitigated differences in glycemic response among bread samples.

In conclusion, our data indicate that the combined effect of gluten and semolina with large particle size resulted in a bread with lower release of reducing sugars during the initial phase of digestion, a reduced insulinemic response and good textural properties.

Keywords:

Coarse semolina; gluten; glycemic index; insulinemic response; oral processing; reducing sugars.

5.1 Introduction

Worldwide, the number of people suffering from type 2 diabetes is around 422 million and this number is continuously rising. It is globally agreed that, by 2025, serious action against the spread of this disease must be taken, also because diabetes is a major cause of the development of cardiovascular diseases (World Health Organization, 2021). The spread of diabetes over the last few decades is attributed to a global increase in obesity, sedentary lifestyle, and the consumption of energy-dense diets, particularly rich in highly digestible starchy foods (Chatterjee et al., 2017). Among highly digestible starchy foods, bread is a staple food daily consumed in Western countries and is characterized by a medium to high glycemic index (Atkinson et al., 2021). Based on a world-recognized classification, carbohydrate-rich foods can be divided into three categories depending on their glycemic index (GI): low, GI < 55, medium, 55 < GI < 69 and high: GI > 70 (Atkinson et al., 2021). This classification reflects how the consumption of food containing carbohydrates affects the postprandial blood glucose level compared to a reference food (such as glucose solution or white bread), which contains the same quantity of available carbohydrates (50 g). High GI foods lead to a significant rise in postprandial blood glucose concentration and, consequently, a high insulin response, potentially leading to hyperinsulinemia and insulin resistance (Stamataki et al., 2017). Therefore, reducing the blood glucose response of starchy foods, like bread, and consequently, its GI, has become of utmost importance.

The leading approach to decrease starch digestibility of bread involves adding viscous fiber, which increases digesta viscosity, slows gastric emptying, and reduces carbohydrate absorption in the small intestine (Rosén et al., 2009; Scazzina et al., 2013). However, adding fiber negatively impacts the textural properties of bread and decreases consumer acceptability (Kurek et al., 2016; Martin et al., 2013). To address this issue, alternative strategies have been explored, such as

limiting starch accessibility to α-amylase through physical barriers naturally present inside the grain (Rovalino-Córdova et al., 2019). In plant-based foods, starch granules are naturally enclosed within cells, protected by a cell wall resistant to digestive enzymes. In legumes, studies have demonstrated that the thick uniform structure of cell walls limits the diffusion of enzymes inside the cells, thereby reducing *in vitro* starch digestibility (Dhital et al., 2016; Rovalino-Córdova et al., 2019). Concerning cereals, *in vivo* and *in vitro* experiments have shown that intact cells can restrict digestive enzyme access to starch in isolated cells, coarse flours containing intact cells (e.g., wheat, sorghum, and barley), and products made from coarse flours, such as porridge (Bhattarai et al., 2018; Edwards et al., 2015a; Korompokis et al., 2019). However, in the case of foods with multiple production steps, like bread, this limiting effect is lost (Korompokis et al., 2021; Tagliasco et al., 2022). The authors hypothesized that during the prolonged mixing time and fermentation step in bread production, the porosity of cell walls increase due to the solubilization of cell wall components like β-glucans and arabinoxylans. This increase in porosity enhances the diffusivity of digestive enzymes within the cell. Additionally, the presence of coarse flour in bread could also reduce the cohesiveness of the crumb, increasing the disintegration rate during digestion and, in turn, the contact surface between the enzyme and its substrate. Therefore, in bakery products the use of coarse flour alone may not efficiently decrease the *in vitro* starch digestibility (Korompokis et al., 2021; Tagliasco et al., 2022).

Gliadin and glutenin, which are the main proteins of wheat grains, after hydration and the application of a mixing force form a discontinuous network that surrounds the starch granules, named gluten (Li, Li et al., 2021). The dense compact gluten network, which is formed during the kneading process of dough, can decrease starch accessibility, acting as a barrier between starch and digestive enzymes (Chen et al., 2019). Substituting a portion of the flour with an increased amount of gluten could also improve the bread structure, enhancing

crumb cohesiveness and resilience. This may, consequently, modulate starch digestibility, limiting the disintegration during the gastric phase and therefore the digestion and absorption of nutrients (Zeng et al., 2023). Differences in bread textures can also impact oral processing and the mastication rate (Lau et al., 2015). It has been recognized that oral behavior partly justifies the individual variations in the glycemic response to foods by affecting the release of starch from the cellular matrix (Gao et al., 2015; Gao & Zhou, 2021; Chen et al., 2023). The relation among food structure, oral processing and glycemic response was demonstrated by comparing foods produced with the same durum wheat flour but with different food textures (i.e., pasta, couscous, and bread) (Vanhatalo et al., 2022). The dense compact structure of pasta, attributed to its robust gluten network, limited disintegration during oral processing and gastric digestion. This led to lower glucose release and, consequently, lower GI compared to bread, which has a more porous easily disintegrated structure (Vanhatalo et al., 2022).

In a preliminary study (Chapter 4), bread produced with coarse semolina, partially replaced with vital gluten, and mixed with the appropriate amount of water in relation to the mixing time, was proven to be the best compromise between good textural properties and lower starch digestibility. This outcome was attributed to the preservation of cell wall integrity, the inhibitory effect of the gluten network on digestive enzymes, and the presence of a cohesive crumb texture. The current study aims to confirm this finding on healthy volunteers, studying the effect of coarse flour and gluten addition in durum wheat bread on the postprandial glycemic and insulinemic responses compared to durum wheat bread made with fine semolina. Furthermore, the oral processing of bread samples was studied to evaluate the effect of gluten and coarse semolina on oral disintegration, the release of reducing sugars after mastication, and, consequently, the effect on glycemic and insulinemic response.

5.2 Materials and methods

5.2.1 Materials

For bread production, durum wheat grains were purchased from Azienda Agricola Bio Nadalautti (Udine, Italy) where two semolina samples with the same proximate composition, but different particle size distribution, were produced: fine semolina (mean particle <400 μm) hereafter indicated as FS, and coarse semolina (mean particle >500 μm) codified as CS. The vital wheat gluten was bought from Primeal (Peaugres, France) and dried yeast (Mastrofornaio PANEANGELI, Cameo s.p.a., Desenzano del Garda, Italy) was purchased from a local supermarket. Monohydrate glucose powder (Farmalabor s.r.l., Canosa di Puglia, Italy) was bought from a local drugstore. The total starch assay kit was bought from Megazyme (Megazyme International, Ireland). All other chemicals and solvents used in the study were of analytical grade.

5.2.2 Methods

5.2.2.1 Test samples preparation

The bread samples were prepared in the experimental kitchen of the Department of Agricultural, Food, Environmental, and Animal Sciences (DI4A) at the University of Udine (Italy). The loaves were produced following the recipe displayed in Table 5.1. To determine the amount of water to add and the required mixing time for a final dough consistency of 500 Brabender units, a water absorption test was conducted at 30 ºC and 63 rpm (1.05 Hz) in a Promylograph T6 (MaxEgger, Austria). Ingredients were mixed for the optimal time (Table 5.1) using a pasta mixer (Pastamatic magnum pro, Simac srl, Mantova, Italia). The resulting dough was placed in a plastic bowl and allowed to ferment at 30 ± 1 °C and 85% relative humidity (RH) for 52 minutes in an incubator (FOC 120i, Velp scientifica, Usmate, Italy).

After this initial fermentation, the dough was manually punched for 2 minutes to redistribute gas bubbles and then molded into a baguette shape, placed in a greased aluminum pan (10 cm × 7 cm × 4 cm), and fermented in the incubator (30 °C – 85% RH) for an additional 50 minutes. At the end of the leavening step, the loaves were baked in a professional oven (A0S101ETA1, 17,5 kW, 400V, 50/60 Hz, Electrolux, Stockholm, Sweden) for 30 minutes at 160 °C, with a relative humidity ramp from 80 to 30%. After baking, bread loaf was removed from the aluminum pan, allowed to cool at ambient temperature (21 ± 1 °C) for an hour and a half, and then stored in a Ziploc® bag (17.7 x 18.8 cm, SC Johnson, United States) overnight. Bread samples were produced freshly the day before each test to standardize the storage time of the product. Each loaf was prepared to contain 50 g of available carbohydrates, determined using the Total Starch kit (Megazyme, Bray, Ireland). The food reference for the glycemic test was prepared by dissolving 55 g of monohydrate glucose powder (Farmalabor s.r.l., Italy) in 250 mL of water. The glucose solution was prepared 24 hours in advance to allow for complete mutarotation of the glucose tautomer (ISO- 26642, 2010). For the oral processing session, bread loaf was sliced into pieces of around 5 g. Due to variations in crumb density among samples, served bread samples had a slightly different volume but the same weight and a consistent proportion between crust and crumb. To prevent moisture loss, the bread samples were prepared just before each oral processing session.

Table 5.1. Formulation of durum wheat bread samples made with fine semolina (FS) and coarse semolina (CS). Ingredients are expressed on 100 g of semolina flour.

	CS (g)	FS (g)	Gluten (g)	Fast action yeast (g)	Salt (g)	Water (g)	Mixing time (min)
95FS_5G	-	95	5	1.2	1	58.5	3.5
80FS_20G	-	80	20	1.2	1	58.5	4.5
80CS_20G	80	-	-	1.2	1	58.5	4.5

FS = fine semolina; CS = coarse semolina; G = gluten.

5.2.2.2 Bread characterization

The volume of the bread samples was evaluated with the rapeseed displacement method following the reported methodology (Method AACC 10-05.01, 2009). Specific volume was calculated by dividing the volume by the weight (cm^3/g). The textural properties of bread samples were assessed with texture profile analysis (TPA) using a TA-XT plus analyzer (Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell and a cylindric aluminum probe of 36.0 mm (P/36). The test conditions followed during the test were: speed of 1.00 mm/s, trigger force of 0.049 N, strain of 40%, and resting time of 5 s. The investigated textural properties were hardness (expressed as the peak force during the first compression, N) and cohesiveness (ratio between the area under the second cycle and the area under the first cycle, dimensionless). For each type of bread, 12 tests were carried out. Images of bread slices were captured using a Canon reflex EOS 550D digital camera (Canon Inc., Tokyo, Japan) with an EF-S 60 mm f/2.8 Macro USM lens. The distance between the bread and the camera lens was consistently maintained at 48 cm, and lighting conditions were carefully controlled. A total of four pictures were taken for each bread recipe. The moisture content of bread was measured following the

official method (Method AACC 44-15.02., 1999). The analysis was performed on an entire slice of bread, with a representative ratio between crumb and crust. The total starch content was measured using the Total Starch assay kit provided by Megazyme (Bray, Ireland), following the procedure for determining the total starch content in samples containing resistant starch. The glucose produced from the enzymatic reaction for total starch was detected through a colorimetric method, with absorbance measured at 510 nm using a Cary 50 spectrophotometer (Agilent Technologies, USA). Protein content was determined using the Kjeldahl method, following the manufacturer's instructions, with a conversion factor of 6.25 for wheat protein (Müller, 2017). Total fiber content was assessed using the Megazyme kit, K-TDRF (Megazyme, Wicklow, Ireland). The fat content was estimated using the fat content of bread ingredients retrieved from the "Food composition database for epidemiological studies in Italy" (BDA; bda-ieo.it) . The total energy calculated for each bread portion was expressed in kcal.

5.2.2.3 Study subjects

Healthy volunteers were recruited through advertisements at the University of Udine. Interested participants attended a preliminary meeting where researchers provided details of the study and discussed potential risks. During this meeting, a physician interviewed participants regarding their general health, medical history, and habitual use of drugs and supplements to assess their eligibility for the study. The exclusion and inclusion criteria were set based on the ISO (ISO-26642, 2010) guidelines with some modifications. The inclusion criteria for participants were as follows: age between 18 and 50 years, overall good health, normal smell and taste functions (self-reported), and a body mass index (BMI) between 18.5 and 24.9 kg/m^2 (based on self-reported weight and height). Exclusion criteria encompassed the presence of dental braces or piercings in or around the mouth (except removable piercings), the use of medications known to affect glucose

tolerance and influence nutrient digestion and absorption (excluding oral contraceptives), a documented history of diabetes mellitus or the use of antihyperglycemic drugs or insulin for diabetes or related conditions, a major medical or surgical event requiring hospitalization in the preceding three months, pregnancy or lactation (self-reported), food allergy or intolerance to gluten, and the use of medications that may affect the function of taste, smell, mastication, and salivation.

5.2.2.4 Study design

The study design is schematically displayed in Figure 5.1. Participants screened for the study, attended all the tests in 6 sessions organized in randomized order determined using the software Smart Sensory Box (Smart Sensory Solutions S.r.l., Sassari, Italy). All the sessions were conducted within a two-month timeframe. The randomized schedule ensured that each participant attended the clinical testing no more than twice a week, with at least a two-day washout period in between. Five sessions were dedicated to the measurement of glycemic and insulinemic response of bread samples (3 sessions) and the glucose standard solution (food reference) (2 times). On these days, the test duration was approximately 2 hours and 30 minutes in total. In an additional session, the participants carried out the oral processing test of the three bread samples during which they were video recorded during bread mastication and the boli were collected. This session had an overall duration of approximately 1 hour. The study was conducted at the Department of Agricultural, Food, Environmental, and Animal Sciences at the University of Udine (Italy). It was approved by the Institutional Review Board of the Department of Agricultural, Food, Environmental and Animal Sciences of the University of Udine (protocol number: 0003800 on July 18th 2023) and was registered at clinicaltrials.gov (NCT06152874). All participants were required to sign the informed consent according to the Helsinki Declaration on human rights.

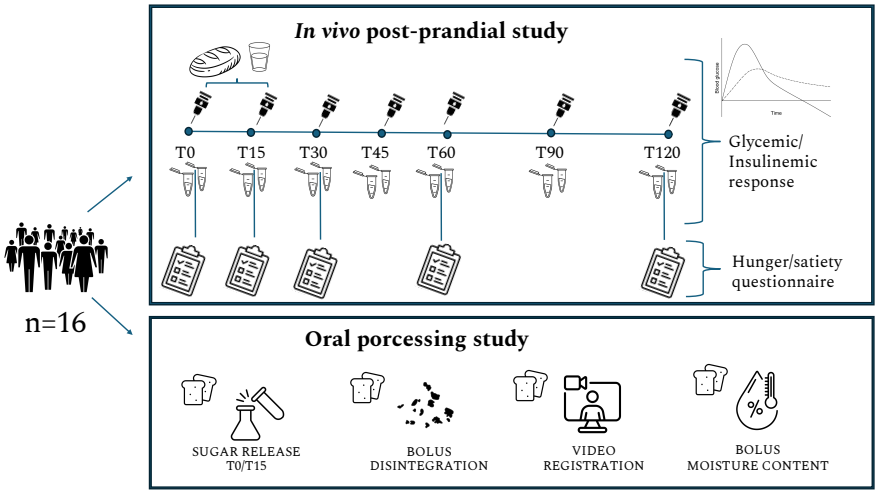


Figure 5.1. schematic overview of the *in vivo* study

5.2.2.5 Postprandial glycemic and insulinemic responses

The analytical protocol for GI determination followed the guidelines set by the ISO (ISO-26642: 2010). Participants were instructed to avoid foods that might interfere with glucose metabolism during the dinner before the test, as reported in the ISO protocol (The instructions to be followed the day before each GI test are displayed in Annex I). Participants arrived at the department at 8 o'clock after overnight fasting and remained seated during the test duration. A fasting capillary blood sample was taken immediately after they arrived, serving as the baseline for blood glucose concentration and plasma insulin concentration. Volunteers were instructed to consume the food within 12 minutes from the first bite (time 0). Bread samples were served along with 250 mL of room-temperature natural water and each subject was asked to drink the same volume for all the tests. Blood samples were taken at 6 time points (15, 30, 45, 60, 90 and 120 minutes) after the participant started consuming the test food. Capillary blood was sampled by finger-prick using a sampling lancet (21G x 1.8 mm, ACCU-CHEK Safe-T-Pro Plus, Roche, Switzerland). At each time

point, blood samples were collected into 2 tubes: four to five drops in one Microvette® CB 300 Fluoride/heparin (SARSTEDT AG & Co., Nümbrech, Germany) for blood glucose analysis and 6-8 drops in one Microvette® CB 300 EDTA K2E (SARSTEDT AG & Co., Nümbrech, Germany) for plasma insulin analysis (Sun et al., 2014). The collected tubes were placed in ice until the end of the test.

5.2.2.5.1 Blood glucose determination

The blood glucose concentration was immediately determined through the YSI 2500 Biochemistry Analyzer (Yellow Springs Instrument Company, Chicago, USA) and expressed in mmol/L. The GI of the bread samples was determined by calculating the incremental area under the curve of the blood glucose (IAUC) resulting from the consumption of each bread sample and expressed as a percentage of the mean IAUC elicited by the consumption of the two-glucose solutions in the same subject.

5.2.2.5.2 Plasma insulin determination

Immediately after the test, the blood collected for insulin measurement was centrifugated at 1970 *g* for 10 minutes at 4 °C and the plasma (around 50-75 µL) was stored at -80 °C until the determination. Insulin concentration in plasma, expressed in milliunits per liter (mU/L), was determined using an immunoassays test kit (Mercodia Insulin ELISA 10-1113-10, Mercodia AB-Uppsala, Sweden), following the instruction of the manufacturer.

5.2.2.5.3 Hunger and satiety ratings during and after the test meal consumption

Hunger and satiety ratings were evaluated with a self-reported questionnaire given to the volunteers to check subjective feelings of fullness and hunger at specific time points (Dall'Asta et al., 2022). The rates of hunger and satiety were assessed at various time points: before the consumption of the food test (fasting), just after food consumption

(t0), and at three subsequent time points during the 2-hour test, after 30 minutes (t30), after 60 minutes (t60), and after 2 hours (t120).

5.2.2.6 Bolus collection and oral processing test

Participants arrived at the session fasting for 2.30 hours, with no smoking or drinking. During this session, participants evaluate a total of 6 cubes for each type of bread. Bread samples were served in a random order and asked to be consumed as a single bite and chewed naturally until the bolus was ready to be swallowed. At that point, the volunteers spitted out the bolus into different containers for further analysis, as follows:

The first and second samples were used to determine released reducing sugars at two time points (t0 and t15). The samples were expectorated into a pre-weighed Falcon conical centrifuge tube. Subsequently, 30 mL of water was given to each participant for mouth rinsing to facilitate the expectoration of any remaining bread particles. The first bolus sample had 1 mL of 1M HCl added immediately after expectoration to block α-amylase activity (t0). The second bolus sample was incubated at room temperature for 15 minutes (t15) and then had 1 mL of 1M HCl added to inhibit α-amylase activity.

The third sample was expectorated into a pre-weighed and pre-dried aluminum pan to determine moisture content (MC%) and saliva content (SC).

The fourth sample was expectorated into a pre-weighed Falcon conical centrifuge tube for the image analysis test. Immediately after expectoration, 30 mL of water was provided to the volunteer to rinse the mouth and remove any remaining particles. At this point, 0.5 mL of 1M HCl was added to the tube to halt the action of salivary α-amylase in the bolus. The bolus was then diluted with an additional 50 mL of water, transferred to a flat plastic container (20.3 cm x 30.5 cm x 5.1 cm), and gently spread with a spatula.

Two additional samples (fifth and sixth) were used for oral processing evaluation.

5.2.2.7 Determination of bolus characteristics and oral processing parameters

1. Release of reducing sugars through the activity of salivary amylase in bolus samples

The quantification of reducing sugars in the bolus, immediately after the chewing (t0) and after 15 minutes of incubation (t15), was carried out following the methodology described by Woolnough et al. (2010) with some modifications. Each bolus (t0 and t15) was centrifuged for 5 minutes at 18630 *g*. Then, 1.5 mL of supernatant was mixed with 0.75 mL of 96 mM 3,5-dinitrosalicylic acid (DNSA) with 1.06 M sodium potassium tartrate. The tubes were incubated for exactly 15 minutes at 100 °C, let cooled down and then diluted with 6.75 mL of distilled water. Reducing sugar absorbance was read at 540 nm using a spectrophotometer (UV-2501PC, UV–VIS Recording Spectrophotometer, Shimadzu Corporation, Kyoto, Japan) (Brodkorb et al., 2019). The values of released reducing sugars at t0 and t15 were expressed in gram of starch per 100 g of total starch of bread sample (% total starch on a wet basis).

2. Moisture and saliva content of bolus

The moisture content of the bolus was measured following the official method (Method AACC 44-15.02., 1999). The bolus was weighed and placed in a 105 °C oven for 16–18 h. After the drying step, samples were cooled in a desiccator for 30 minutes and subsequently weighed. Bolus moisture content (MC%) on a wet weight basis was calculated using the following equation $MC\ (\%) = (m_0 - m_1)/m_0 \cdot 100$, where m_0 is the weight of the sample before drying and m_1 is the weight after drying. Bolus moisture content on a dry weight basis was calculated using the

following equation $MC_{db} = (m_0 - m_1)/m_1$. Saliva content (SC) per gram of dry food was determined by subtracting the moisture content on a dry-weight basis of the bread sample from the moisture content on a dry-weight basis of the bolus. The calculation assumed that the bolus was completely expectorated.

3. Particle size of bolus

The particle size of the bolus was measured using image analysis. The bolus spread on the flat plastic container was scanned using a printer (Kyocera, Kyoto, Japan) and analyzed with ImageJ (ImageJ, Bethesda, Maryland, USA), following the method described by Chen et al. (2021). The captured images were transformed into 8-bit and adjusted for brightness/contrast with a threshold of 50-255. For each image, particles smaller than 0.03 mm^2 were excluded from data analysis to prevent interference with the background. The mean area (mm^2) of the bolus particles was reported for each bread sample.

4.Oral processing parameters

To record the mastication behavior, subjects were comfortably seated in a chair in front of a desk equipped with a Logitech C920 PRO HD webcam (Logitech Europe S.A, Losanna, Switzerland) (resolution 1080p/30fps), placed approximately 50 cm from the participant's face. Participants were instructed to stand straight in front of the camera, avoiding covering their mouth and neck with their hands during mastication. A glass of water was provided with the testing samples to rinse the mouth between each bread bite. The oral processing parameters were extracted manually from video recordings and the parameters analyzed were: the number of chews and the total eating duration expressed in seconds (s). The eating rate, expressed as the amount of food consumed per minutes (g/min), was then calculated.

The parameters were expressed for each subject as the mean of the two bread bites (Mosca et al., 2022).

5.2.3 Statistical analysis

The number of volunteers to be involved in the study was determined through a power analysis using the incremental area under the curve of glucose release (mmol/L x min) as a bench marker. Based on the post-hoc analysis of the study of Chiavaroli et al. (2021), it was found that 12 volunteers were sufficient to ensure a test power of 80% and an α error of 0.05. These parameters allowed the detection of differences in the incremental area under the curve of glucose release assuming an average value of 76 mmol x min/L and a standard deviation (SD) of 12. Considering a possible 25% dropout rate, the number of participants recruited was set at 16. For the textural characteristics of bread (i.e., specific volume, hardness, and cohesiveness), the statistical method used to determine the significant differences among the analyzed samples was one-way ANOVA ($p < 0.05$) followed by a post-hoc Tukey's test ($p \leq 0.05$). These data were expressed as means ± standard deviation (SD). The IAUC of glucose, insulin, decrease of hunger and increase of satiety was geometrically calculated using the trapezoid rule, ignoring the area beneath the baseline, as reported by ISO guidelines (ISO-26642, 2010). All data collected from the *in vivo* test were assessed for normality using the Kolmogorov-Smirnov test, then presented as mean ± standard error of the means (SEM) and analyzed using the general linear model for repeated measures. Greenhouse–Geisser correction for degrees of freedom was applied when the Mauchly test of sphericity was significant. Significant differences among the analyzed samples were determined using the Bonferroni post-hoc test for multiple comparisons ($P < 0.05$) (Vanhatalo et al., 2022). All the statistical analyses were conducted using IBM SPSS Statistics for Windows version 28.0 (IBM Corp., Armonk, N.Y., USA).

5.3 Results

5.3.1 Textural and nutritional characteristics of bread samples

To produce the reference bread made with fine durum wheat semolina (i.e., 95FS_5G), the inclusion of 5% gluten was necessary due to the poor gluten functionality of the used semolina.

The textural characteristics of bread samples are displayed in Table 5.2. The 95FS_5G sample was characterized by the lowest specific volume, the highest hardness and was the least cohesive among the analyzed samples. Textural properties of 80FS_20G and 80CS_20G samples were not significantly different. Representative images of slices and bread loaf sections are displayed in Figure 5.2. The crumb of the three bread slices appeared uniform and compact. In the 95FS_5G bread, the loaf structure was flatter compared to the other bread samples, which exhibited a more expansive porous structure. In the 80CS_20G sample, produced with coarse semolina (particle size > 500 μm), intact particles were detectable in the crust. The nutritional composition of the bread samples per serving consumed during the tests is displayed in Table 5.3. The carbohydrate content was around 50 g and consistent for each sample, as required by the ISO guidelines (ISO-26642, 2010) for GI determination. Samples 80FS_20G and 80CS_20G contained a higher amount of protein and a slightly higher fat content compared to 95FS_5G bread. This higher amount of protein and fat led to a higher content of total energy in 80FS_20G and 80CS_20G compared to the other bread.

Table 5.2. Specific volume, hardness and cohesiveness of bread samples made with fine or coarse semolina partly substituted with 5% or 20% gluten.

	Specific volume (cm^3/g)	**Hardness (N)**	**Cohesiveness (-)**
95FS_5G	2.17 ± 0.11^b	53.45 ± 0.76^a	0.67 ± 0.02^b
80FS_20G	3.25 ± 0.14^a	7.02 ± 0.91^b	0.84 ± 0.00^a
80CS_20G	2.94 ± 0.11^a	8.20 ± 0.13^b	0.85 ± 0.01^a

Mean values (n=12) within a column with different letters were significantly different ($p< 0.05$; ANOVA test followed by Tukey's test). FS = fine semolina; CS = coarse semolina. G=gluten. The data were expressed as means ± standard deviation.

Figure 5.2. Representative images of the whole bread samples and the respective slices of bread samples made with fine or coarse semolina partly substituted with 5% or 20% gluten. FS = fine semolina; CS = coarse semolina; G=gluten.

Table 5.3. Nutritional composition of the serving of bread samples made with fine or coarse semolina partly substituted with 5% or 20% gluten.

	Bread portion (g)	**Energy (kcal)**	**Carbo-hydrate (g)**	**Protein (g)**	**Fiber (g)**	**Fat# (g)**	**Water content (g)**
95FS_5G	109.5 ± 1.7^{b}	267.3 ± 4.0^{b}	49.8 ± 0.8^{a}	9.9 ± 0.1^{b}	4.7 ± 0.1^{a}	2.1	34.7 ± 0.5^{b}
80FS_20G	135.2 ± 1.2^{a}	314.5 ± 2.3^{a}	50.4 ± 0.4^{a}	20.4 ± 0.2^{a}	4.7 ± 0.0^{a}	2.6	43.0 ± 0.3^{a}
80CS_20G	133.1 ± 1.9^{a}	311.6 ± 3.9^{a}	49.3 ± 0.7^{a}	20.1 ± 0.1^{a}	4.6 ± 0.1^{a}	2.5	42.9 ± 0.9^{a}

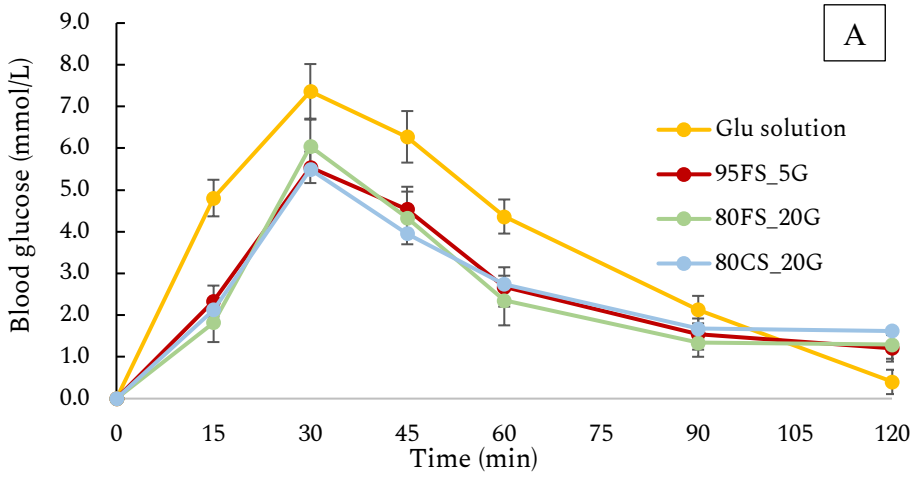
The data were expressed as means ± standard deviation. Mean values of bread samples (n=3) within a column with different letters were significantly different ($p < 0.05$; ANOVA test followed by Tukey's test). #The fat content was estimated using the fat content of bread ingredients retrieved from the "Food composition database for epidemiological studies in Italy" (BDA; bda-ieo.it). FS = fine semolina; CS = coarse semolina. G=gluten.

5.3.2 Study participants

Twenty-two participants expressed interest in the study, 18 were screened and met the inclusion criteria, and 16 volunteers agreed to participate and completed the study without any protocol violations. The participants, consisting of 10 females and 6 males, had an average age of 28.8 ± 2.2 years, an average weight of 66.5 ± 12.8 kg, an average height of 1.74 ± 0.10 m, and an average body mass index of 21.8 ± 2.1 kg/m^2. All the values are expressed as mean ± SEM.

5.3.3 Postprandial blood glucose responses

Postprandial blood glucose response curves, incremental area under the curve over 2 hours ($IAUC_{120}$), and peak glucose at t=30 of analyzed bread samples and glucose solution are displayed in Figure 5.3. The $IAUC_{120}$ value for glucose (196.5 ± 14.9 mmol/L x min) was significantly higher than that of the three bread samples. Bread samples induced similar $IAUC_{120}$, which ranged between 132.9 ± 11.8 mmol/L x min for 80FS_20G sample and 139.9 ± 13.5 mmol/L x min for 95FS_5G sample. The glucose concentration peaked after 30 minutes for all samples without any significant difference and ranged from 7.4 ± 0.3 mmol/L for glucose solution to 5.5 ± 0.6 mmol/L for 80CS_20G bread. GI values were 73.7 ± 11.1 for 80FS_20G sample, 72.9 ± 9.8 for 80CS_20G sample, and 71.9 ± 6.4 for 95FS_5G sample. No significant differences in GI were observed among them. According to the GI classification (Atkinson et al., 2021), all three bread samples can be categorized as high GI (GI > 70).



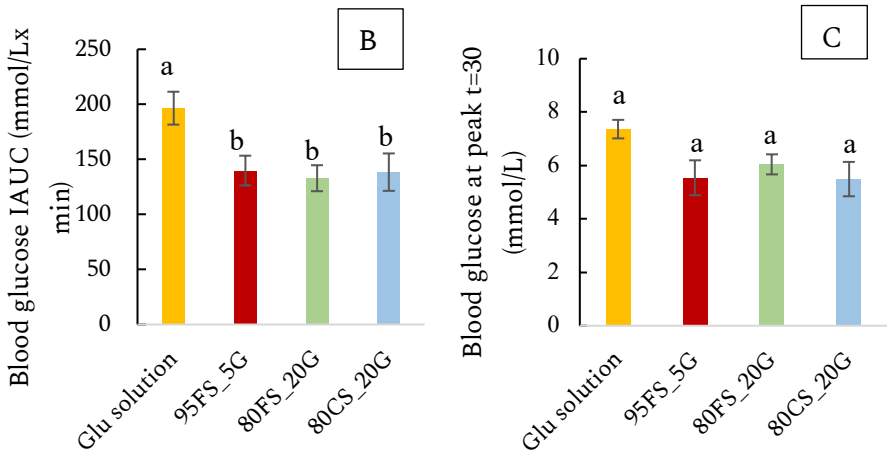


Figure 5.3. Incremental blood glucose curves (A), incremental areas under curves at 0-120 minutes ($IAUC_{120}$) (B) and blood glucose incremental peak (t=30) (C) after consumption of glucose solution and the three bread samples made with fine or coarse semolina partly substituted with 5% or 20% gluten. FS = fine semolina; CS = coarse semolina; G = gluten; glu = glucose. Values are presented as the means ± standard error of the mean (n = 16). Columns sharing the same letter were not significantly different (General linear model for repeated measures followed by post-hoc test Bonferroni $p< 0.05$).

5.3.4 Postprandial plasma insulin responses

Figure 5.4 shows postprandial plasma insulin response curves over 2 hours, along with $IAUC_{120}$ and peak insulin concentration at t=30, of analyzed bread samples and glucose solution. $IAUC_{120}$ values ranged from 5519.9 ± 699.5 mU/L x min for 95FS_5G sample to 7480 ± 1199.3 mU/L x min for 80FS_20G sample. However, no significant differences were detected among the analyzed samples and the glucose solution. The plasma insulin incremental peak was observed for all three analyzed samples after 30 minutes of bread consumption. The peak concentration of plasma insulin elicited by 80FS_20G sample (37.3 ± 3.5 mU/L) was significantly higher than that of 80CS_20G sample (26.6 ± 3.4 mU/L) and 95FS_5G bread (26.6 ± 3.4 mU/L). The glucose solution elicited an insulin response similar to that of the other bread samples.

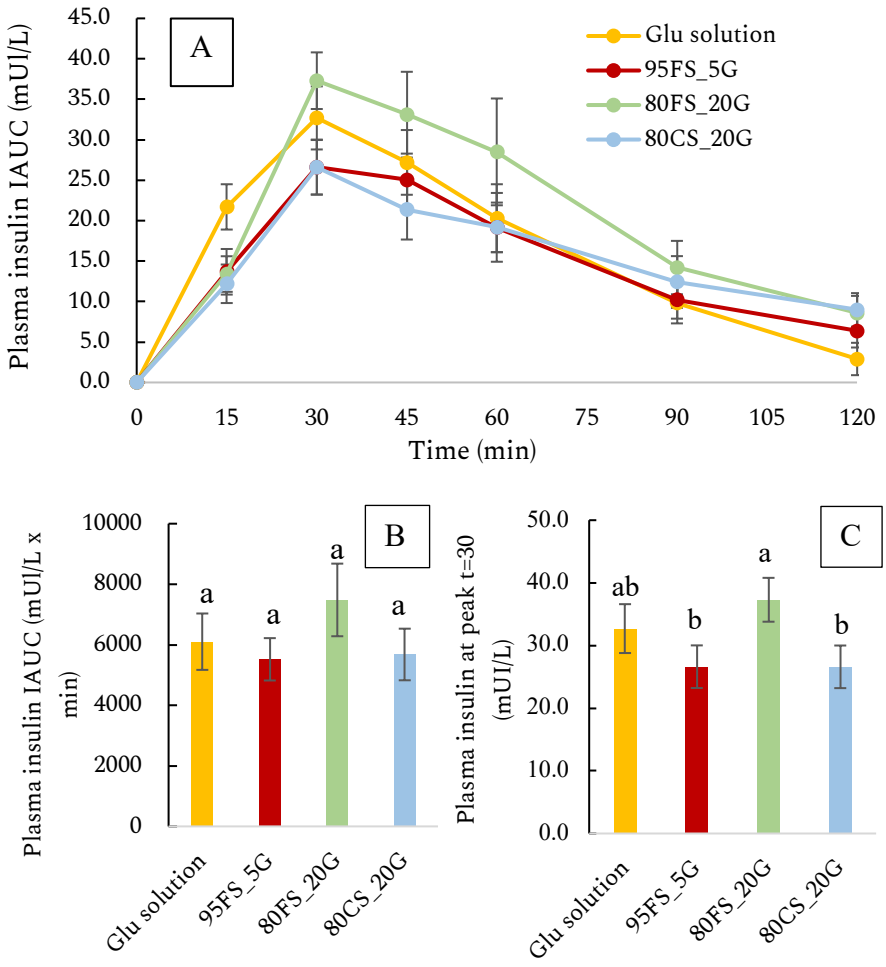


Figure 5.4. Incremental plasma insulin curves (A), plasma insulin incremental area under curves at 0-120 minutes ($IAUC_{120}$) (B) and plasma insulin incremental peak (t=30) (C) after consumption of glucose solution and the three bread samples made with fine or coarse semolina partly substituted with 5% or 20% gluten. FS = fine semolina; CS = coarse semolina; G = gluten; glu = glucose. Values are presented as the means ± standard error of the mean (n = 16). Columns sharing the same letter were not significantly different (General linear model for repeated measures followed by post-hoc test Bonferroni $p < 0.05$).

5.3.5 Ratings of hunger and satiety

The rates of increase in satiety and reduction in hunger, along with the corresponding IAUC over 120 minutes of the test, are presented in Figure 5.5. The increase in satiety and decrease in hunger did not show significant differences among the three bread samples; however, the increase in satiety and the reduction in hunger were significantly lower after the consumption of the glucose solution than after bread consumption.

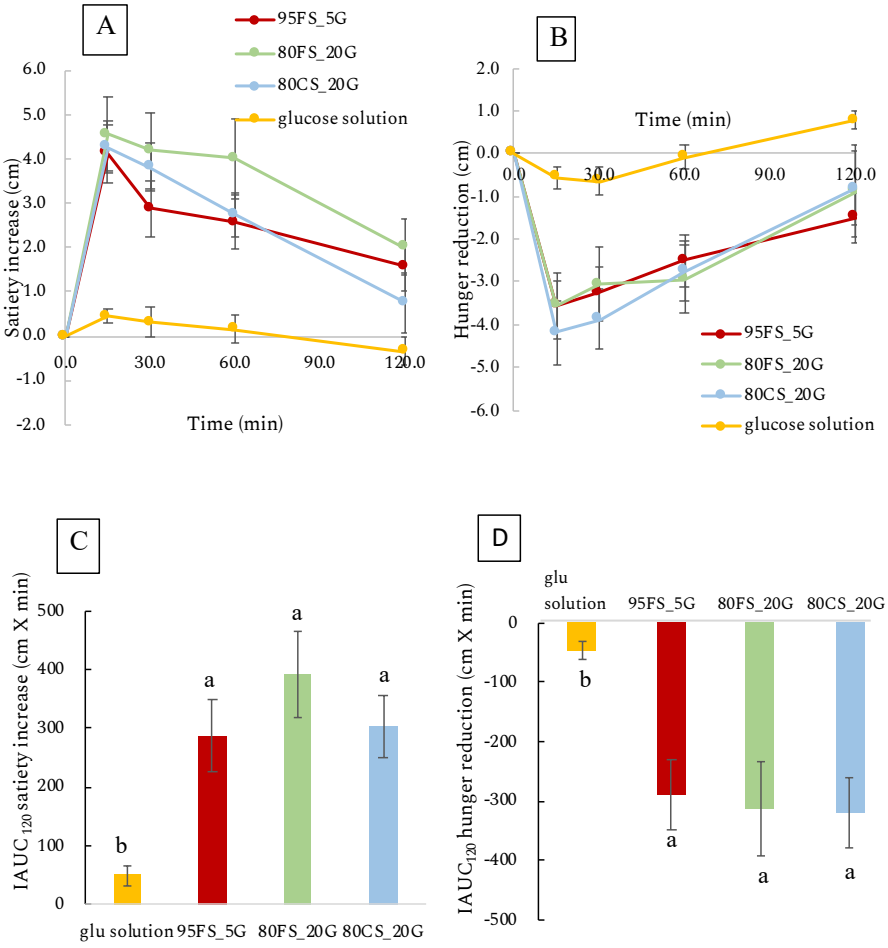


Figure 5.5. Increase of satiety over time (A), decrease of hunger over time (B), $IAUC_{120}$ of satiety increase (C) and $IAUC_{120}$ of hunger reduction (D) after consumption of glucose solution and the three bread samples made with fine or coarse semolina partly substituted with 5% or 20% gluten. FS = fine semolina; CS = coarse semolina; G=gluten; glu = glucose. Values are presented as the means ± standard error of the mean (n = 16). Columns sharing the same letter were not significantly different (General linear model for repeated measures followed by post hoc test Bonferroni $p < 0.05$).

5.3.6 Bolus properties

Among the three samples, the moisture in the bolus was significantly higher in 80CS_20G bread than in 80FS_20G and 95FS_5G sample, whereas the saliva content in the bolus did not exhibit any significant differences among the three bread recipes (Table 5.4). The mean area of bolus particles and the released reducing sugars in the bolus at t0 and t15 are depicted in Figure 5.6. The area of the bolus particles expectorated after the consumption of 80FS_20G sample was significantly larger than that of 95FS_5G sample, while the area of 80CS_20G sample did not significantly differ from that of the other two bread samples. The amount of reducing sugars released in the bolus after the consumption of 80FS_20G sample at t0 was significantly higher than the amount released from 80CS_20G sample. At t15, both 80CS_20G and 95FS_5G samples released significantly fewer reducing sugars in their boli than the amount released in 80FS_20G bread.

Table 5.4. Moisture content of bolus and saliva content of bread samples made with fine or coarse semolina partly substituted with 5% or 20% gluten.

	Bolus moisture content (%, on wet basis)	**Saliva content (g/100 g dry matter)**
95FS_5G	71.2 ± 0.9[a]	8.9 ± 1.9[a]
80FS_20G	71.3 ± 0.8[a]	9.3 ± 1.7[a]
80CS_20G	72.4 ± 0.9[b]	9.5 ± 2.0[a]

Values are expressed as mean ± standard error of the mean. Mean values (n=16) within a column with different letters were significantly different (General linear model for repeated measures followed by post-hoc test Bonferroni $p < 0.05$). FS = fine semolina; CS = coarse semolina; G=gluten

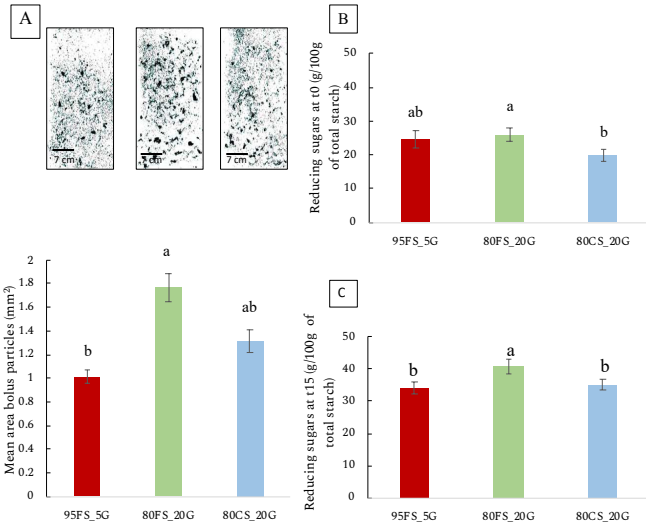


Figure 5.6. Mean area of bolus particles with representative scans of separated boli particles of one subject on the top (A), released reducing sugars in the bolus at t0 (B) and released reducing sugars in the bolus at t15 (C) of the analyzed bread samples made with fine or coarse semolina partly substituted with 5% or 20% gluten. FS = fine semolina; CS = coarse semolina; G = gluten. Values are presented as the means ± standard error of the mean (n = 16). Columns sharing the same letter were not significantly different (General linear model for repeated measures followed by post-hoc test Bonferroni $p < 0.05$).

5.3.7 Oral processing parameters

The number of chews, total eating duration, and eating rate are presented in Table 5.5. The number of chews ranged from 41.1 ± 4.2 for 95FS_5G sample to 46 ± 4.9 for 80FS_20G sample. The total eating duration was approximately 34 s, and the eating rate was 9.5 g/min. No significant differences in terms of all the oral processing parameters were found among the three bread samples.

Table 5.5. Number of chews, total eating duration and eating rate of bread samples made with fine or coarse semolina partly substituted with 5% or 20% gluten.

	Number of chews	**Total eating duration (s)**	**Eating rate (g/min).**
95FS_5G	41.1 ± 4.2[a]	32.5 ± 4.2[a]	10.6 ± 0.9[a]
80FS_20G	46.2 ± 4.9[a]	35.2 ± 3.4[a]	9.3 ± 0.6[a]
80CS_20G	44.2 ± 4.0[a]	34.9 ± 2.6[a]	9.3 ± 0.6[a]

Values are expressed as mean ± standard error of the mean. Mean values (n=16) within a column with different letters were significantly different (General linear model for repeated measures followed by post-hoc test Bonferroni $p < 0.05$). FS = fine semolina; CS = coarse semolina; G = gluten;

5.4 Discussion

Consuming a low-GI diet instead of a high-GI diet is considered effective in reducing the risk of major non-communicable diseases, including type 2 diabetes, cardiovascular disease, and colorectal cancer (Fan et al., 2012; Livesey et al., 2019; Turati et al., 2019). Reducing the GI of bread, a significant contributor to the glycemic load in Western diets (Rodríguez-Rejón et al., 2014), represents a promising strategy for lowering the overall GI of the diet. In the present study, we reported the effects of coarse semolina and gluten in bread, and the consequential modulation in texture, on the postprandial glycemic and insulinemic responses in healthy volunteers. We also investigated the oral processing of bread samples to understand how gluten and particle sizes of semolina influence oral disintegration, the release of reducing sugars after mastication, and subsequently, the glucose and insulin responses. We expected the consumption of bread made with coarse semolina partly substituted with 20% gluten, i.e., 80CS_20G sample, would result in a low glycemic response due to lower digestion of starch in the coarse semolina rich in intact cells and the addition of gluten, which makes dense compact network able to decrease the digestive enzyme accessibility and a cohesive bread structure.

The presence of semolina large particles in bread has a detrimental effect on the textural properties of the crumb by increasing the hardness and reducing the volume and cohesiveness (Bressiani et al., 2017; Korompokis et al., 2021). During digestion, bread with a high cohesiveness will rapidly disintegrate leading to quick starch hydrolysis, compared to bread that better preserves its structure (see chapter 3), and counterbalancing the effect of intact cell walls in limiting starch hydrolysis (Korompokis et al., 2021). Therefore, we substituted 20% semolina with vital gluten to improve crumb cohesiveness and the overall textural properties of sample made with coarse semolina. This substitution was effective since the bread made

with coarse semolina (i.e., 80CS_20G) exhibited similar textural properties as that made with fine semolina (i.e., 80FS_20G). However, despite the difference in semolina particle size, no differences in glycemic response were found between the two samples (80CS_20G and 80FS_20G). Interestingly, 80CS_20G triggered a significantly lower insulin production 30 minutes after bread consumption compared to the counterpart made with fine semolina. Similarly to our findings, Eelderink et al. (2017) observed that the incorporation of cracked kernels (containing clusters of intact cells) significantly decreased insulin response compared to a control bread, without impacting the glycemic response. As reported by several authors, the mechanism behind the differences in insulinemic response can be explained by different flows of sugar entering the duodenum that modulate insulin production (Eelderink et al., 2017; Pentikäinen et al., 2014).

After mastication, once the bolus enters the stomach, salivary α-amylase remains active until the bolus is fully disintegrated and the chyme pH does not drop down to 5 thanks to HCl production. Consequently, starch hydrolysis persists for a longer time, ranging between 15 and 30 minutes, after the bread has been swallowed depending on its bolus properties, releasing maltose and maltodextrins and oligosaccharides (Freitas et al., 2018, 2022; Woolnough et al., 2010). Therefore, to understand how the differences in bolus properties may have affected the post-prandial glucose release, the amount of reducing sugars released in the bolus was measured right after mastication and after 15 minutes of incubation, to estimate the amount of partially hydrolyzed starch entering the intestine. Although the bread samples were similarly chewed, the bolus produced after consumption of 80CS_20G contained a significantly lower amount of reducing sugars than that of 80FS_20G, both immediately after mastication and after 15 minutes of incubation. The presence of intact cell clusters in bread 80CS_20G may have reduced the sugar release at t0 and t15. In bread produced

with coarse semolina, the presence of intact cells was detected even after the baking process (Korompokis et al., 2021; Tagliasco et al., 2022), and the cell integrity might have hindered starch accessibility during the oral phase and the early gastric phase (Edwards et al., 2015a), limiting the amount of reducing sugars released and, consequently, insulin production. Conversely, boli expectorated from bread made with fine semolina released more reducing sugars at t0 and t15. As reported by several authors (Röder et al., 2016; Pentikäinen et al., 2014; Yabe & Seino, 2011), a higher amount of glucose released after oral processing could induce a higher production of glucose-dependent insulinotropic peptide (GIP), stimulated by the flow of glucose to the duodenum, which, subsequently, stimulates insulin secretion from pancreatic β cells. We hypothesized that the differences in insulin production, triggered by the various amounts of reducing sugar released after oral processing, could have potentially mitigated differences in glycemic response among bread samples.

Besides cell intactness, bread structure may also have had an effect in modulating insulin release. This is clear when bread produced with fine semolina and a low substitution of gluten (95FS_5G) is compared to that produced with the same semolina but a higher substitution of gluten (80FS_20G). Despite the similarities in glycemic responses, the former bread elicited a significantly lower insulin production at 30 minutes compared to the latter one. This sample (95FS_5G), in contrast to the other analyzed bread samples, exhibited a significantly lower specific volume and cohesiveness, and a higher hardness, due to the lower content of gluten. Consistent with these findings, Nordlund et al. (2016) observed an increase in insulin response after the consumption of wheat bread, characterized by a porous structure, compared to rye bread, which had a dense compact crumb. In agreement with the present study, they did not observe differences in glycemic response. In addition, after mastication, we observed that the hard compact bread (95FS_5G) produced a bolus with smaller particles

and released a lower amount of reducing sugars compared to soft, porous bread (80FS_20G). This is consistent with the findings of Pentikäinen et al. (2014), who reported that even if rye bread was chewed into smaller particles, its compact structure resulted in the release of a slightly lower amount of sugars compared to wheat bread, characterized by larger bolus particles and a porous structure. The same authors further confirmed this, demonstrating that tri-, tetra-, and monosaccharides were released to a greater extent in saliva after the mastication of porous wheat bread than from compact rye bread (Pentikäinen et al., 2019). Therefore, in the case of 80FS_20G sample, its porous structure and the use of fine semolina may have facilitated the access of salivary α-amylase to starch granules, leading to faster starch hydrolysis with a consequent high sugar release at the oral level. In contrast, the compact structure of 95FS_5G bread may have delayed starch hydrolysis in the oral phase by restricting α-amylase accessibility to starch and leading to a lower insulin response (Eelderink et al., 2015). The differences in insulinemic response between 80FS_20G and 95FS_5G can also be attributed to the different amounts of protein in the two samples. As reported by several authors, proteins can have an insulinotropic effect and enhance insulin production after the consumption of protein-enriched foods. This is due to the presence of free amino acids, formed after gastric digestion, which can stimulate insulin release by pancreatic β cells (Adams & Broughton, 2016; Sun et al., 2014; Van Loon et al., 2000). However, no differences were found in insulin response between 80CS_20G and 95FS_5G, even though 80CS_20G had a higher amount of protein, comparable to the amount present in 80FS_20G. The insulinotropic effect of the protein added in samples 80CS_20G was probably counterbalanced by the presence of clusters of intact cells that delayed glucose release and therefore insulin production. The presence of a fourth sample made with coarse semolina and 5% gluten would have been useful to underline and isolate the effect of protein addition on the insulin response of bread made with coarse semolina.

This study presents several strengths and some limitations. Firstly, all tested samples were produced following a standardized procedure the day before the test to ensure a consistent storage period. Moreover, all the bread samples were produced starting from the same durum wheat semolina and milled at different extents to obtain various particle sizes, therefore having the same content of fiber.. Only gluten, and no other potentially interfering ingredients like fat, was used to modulate the textural properties of bread samples. Furthermore, the glycemic response measurement was coupled with the insulinemic response to better understand the metabolic responses elicited by bread consumption. Additionally, the mastication step was included to study the relationship between bread structural properties and starch digestibility and its effect on postprandial responses. The main limitation concerns the absence of a fourth sample produced with coarse semolina and 5% gluten to better understand the role of semolina particle size in affecting the glycemic and insulinemic responses and isolate the effect of protein addition. Unfortunately, this sample could not be produced due to the lack of a strong gluten network able to entrap the semolina coarse particles in the bread structure. Moreover, the quantification of incretins (e.g., GIP), which affect insulin release, could have been useful to confirm the effect of the first phase of starch hydrolysis and sugar release on the insulin response. Finally, the lack of control for the menstrual cycle in female volunteers is another limitation of this study.

In conclusion, our data indicate that the combined effect of gluten and semolina with large particle size (80CS_20G) resulted in a lower release of reducing sugars during the initial phase of starch hydrolysis, which may have been responsible for the reduced insulinemic response compared to its counterpart made with fine semolina (80FS_20G). Our results also showed that a compact bread structure (95FS_5G) can elicit a lower insulinemic response compared to its more voluminous counterpart. However, consumers generally prefer a bread with a soft porous structure (Angioloni & Collar, 2009; Moretton

et al., 2023; Żakowska-Biemans & Kostyra, 2023). Therefore, a bread made with a combination of coarse semolina and 20% gluten substitution represents a good compromise between good textural properties and a reduced insulinemic response.

ANNEX I

Instructions to be followed the day before each GI test

- No food or drink other than water for 12 h or more before the test;
- No vigorous exercise in the morning of the test and the day before;
- No smoke in the morning;
- No drugs the day before the test (except the following drugs i.e., anti-inflammatory, antibiotics and antiviral, taken in case of need), the subjects will be asked to report this to the researcher who is carrying out the test;
- Consume the same meal the dinner before each test. NOT including the following food products:

    - Whole products (bread, snacks, cereal, etc.).
    - Pasta in general; better to consume white rice or pizza.
    - Cold potatoes or canned purée; better to eat salad or tomatoes as a side dish.
    - Legumes (beans, chickpeas, lentils, soy products).
    - Fruits (except ripped bananas, oranges, or mandarins).
    - Cloudy juices.
    - Drinks and candies sweetened with sorbitol/xylitol.
    - Alcohol (no more than a glass of wine or a small beer).

General discussion

6.1 Brief introduction

This Ph.D. project aimed to investigate, from micro- to macro-structure, the effect of physical barriers, such as cell walls, protein matrix, and food texture, and their interactions, on the starch digestibility of different bread products.

In this chapter, a detailed discussion is provided on the effects of each physical barrier on starch digestibility of bread, going step by step.

Additionally, this chapter offers an overview of the main findings of this thesis, a critical discussion of the principal methodologies utilized and a brief resume of the future perspective in this field.

An overall summary of the main findings of this thesis is presented in Table 6.1.

Table 6.1: Summary of the aim and the main results of the projects developed in the thesis

Aim	Main results
• Check the presence and assess the impact on starch digestibility of intact cell clusters in flour and bread made from durum wheat and rye, produced with increasing particle sizes: small (<350 µm), medium (1000 µm-1800 µm), and large (>1800 µm). (**Chapters 2-3**)	• Clusters of intact cells were detectable through microscopic analysis in flours (>1000 µm) and bread made with those durum wheat and rye flours. • The presence of intact cells can limit starch digestibility in durum wheat and rye flour but not in bread.
• Monitor the ability of cell walls to limit starch digestibility throughout all the stages of the baking process, from raw flour to dough and bread. (**Chapter 2**)	• After the kneading step, clusters of intact cells were still detectable in the dough, but they had lost their ability to limit starch digestibility.
• Study the effect of increasing particle sizes of flour (small <350 µm, medium 1000 µm-1800 µm, and large >1800 µm) on the textural properties of durum wheat and rye bread. (**Chapters 2-3**)	• Increasing the particle size of flour results in a decrease in the volume and cohesiveness and an increase in hardness in durum wheat and rye bread.
• Evaluate the physical disintegration of bread during digestion to study the relationship between the integrity of cell walls, the structural features of bread, and *in vitro* starch digestibility. (**Chapter 3**)	• Bread produced with coarse flour exhibited the least cohesive and resilient texture, disintegrated more during digestion, and had the highest starch digestibility.

• Elucidate the effects of dough mixing time, gluten addition, and dough hydration on modulating the food matrix, texture and starch digestibility of durum wheat bread made with coarse semolina (>1000 µm). (**Chapter 4**)	• A 20% gluten substitution, coupled with the right amount of water in the dough, improves the textural qualities of bread made with coarse semolina. • When 20% coarse semolina was substituted with gluten, the amount of starch digested after 120 minutes was reduced.
• Test the glycaemic and insulinemic response of bread made with 80% coarse semolina (>500 µm) and 20% gluten compared to bread made with fine semolina (<400 µm) and 20% gluten, and bread made with 95% fine semolina and 5% gluten. • Evaluate mastication behavior and oral disintegration of bread samples, and release of reducing sugars in the boli after mastication. (**Chapter 5**)	• A 20% gluten substitution enhances the textural properties of bread made with coarse semolina. • The combined effect of coarse semolina and gluten addition slows down the release of reducing sugars in the boli after mastication, eliciting a lower amount of insulin 30 minutes after bread consumption. • Bread made with 95% fine semolina and 5% gluten had a compact structure which released a lower amount of reducing sugars in the boli after mastication and elicited a lower amount of insulin than its counterpart made with a higher amount of gluten, which was characterized by expansive volume and crumb. • No differences were detected in the glycemic responses among the three bread samples.

6.2 Effect of physical barriers on the starch digestibility of bread products

6.2.1 Cell wall integrity

In plant foods, starch granules are naturally encapsulated within cells and this natural physical barrier has been shown to reduce digestibility by slowing down the contact between the starch and digestive enzymes (Rovalino-Córdova et al., 2018, 2019). This mechanism has been extensively studied in legumes, which typically have thick, uniform cell walls (Dhital et al., 2016; Rovalino-Córdova et al., 2019). Less clear is whether this barrier effect is present also in cereals. Studies on isolated cells from wheat and sorghum have shown that starch digestibility is significantly lower in intact cells compared to damaged ones (Bhattarai et al., 2018; Korompokis et al., 2019). However, when coarse flour, rich in clusters of intact cells, is used to produce bread, no effect in modulating the rate of starch digestibility was observed (Korompokis et al., 2021).

Chapters 2 and **3** of this thesis focused on the impact of intact cell walls in rye and durum wheat grains on starch digestibility. To create flours with varying amounts of intact cell wall clusters, the kernels were mildly milled to produce medium (1000 μm-1800 μm) and large (>1800 μm) particles flours, and then re-ground to obtain small (<350 μm) flour. Confocal laser scanning microscopy revealed clusters of intact cells in flour fractions with particle sizes larger than 1000 μm in both cereals, while cells appeared predominantly damaged in flour with particle sizes smaller than 350 μm. *In vitro* starch digestibility of flour decreased with increasing particle size. However, in bread, even though intact cells were still detectable, no significant difference in starch digestibility was observed. In **Chapter 2**, the study monitored

starch digestibility at various stages of the baking process, along with the detection of intact cells using CLSM, to identify the stage at which the physical encapsulation of starch within cell walls loses its effectiveness in reducing *in vitro* starch digestibility. The results clearly showed that after the kneading and fermentation, the presence of clusters of intact cells was no longer able to modulate the starch digestibility, even though intact cells were still detectable in dough made with medium and large particle size flours. Therefore, we hypothesized that, during the long mixing time (60 and 90 minutes, respectively for the medium and large flour) and fermentation steps, the porosity of the cell walls increased due to the solubilization of the main components of the wheat cell wall. Therefore, in **Chapter 4**, we explored the effect of long *versus* short mixing time on the starch digestibility of bread made with coarse semolina to indirectly investigate the impact of mixing time on cell wall porosity. In this chapter, bread made with coarse semolina and 20% gluten substitution and a 3.5-minute mixing time was compared to its counterpart mixed for 45 minutes. No significant differences were detected in starch digestibility between these two types of bread indicating that the mixing time does not affect starch digestibility. Therefore, all bread preparation steps (kneading, fermentation, baking) might have contributed to an increase in porosity. However, a direct measurement of cell wall porosity would have been valuable for a deeper understanding of this mechanism (refer to methodological considerations).

In **Chapter 5**, we evaluated the effect of coarse semolina on postprandial glycemic and insulinemic responses, also including the oral processing and the release of reducing sugars in the boli after mastication, in healthy participants. The results show that, although no differences in the glycemic responses were detected, the presence of intact cell walls significantly decreased the release of reducing sugars in the boli after mastication and after a 15-minute incubation at room temperature. This low release of reducing sugars in the oral

phase may explain the lower insulin response after 30 minutes of the consumption of bread made with coarse semolina compared to that made with fine semolina. Interestingly, contrary to the findings reported in **Chapters 2** and **3**, the clusters of intact cell walls were able to act as a barrier between starch and salivary α-amylase, reducing hydrolysis, at least during the oral phase. We hypothesized that, due to the short duration of mastication, approximately 34 seconds, the presence of intact cells can effectively slow down starch digestibility, limiting the contact between the encapsulated starch and enzymes. However, during the entire digestion process, the porosity of the cell walls in the bread matrix may increase significantly, allowing enzymes to penetrate the cells and hydrolyze the starch. This finding differs from what was observed by Edward et al. (2015), who found still unhydrolyzed starch entrapped in cells of coarse wheat porridge at the end of digestion in ileostomy patients. This different outcome between our study and Edward's might be due to a more detrimental effect of the baking process on the wheat cell wall, which probably increased dramatically cell wall porosity, compared to the cooking step in porridge preparation.

In conclusion, the integrity of cell walls in cereals can effectively reduce starch digestibility in bread made with coarse semolina during the early stages of digestion, but the effect is lost through the entire digestion process.

6.2.2 Gluten substitution

Gliadin and glutenin, the main storage proteins of wheat, after hydration and mixing force form a discontinuous network known as gluten (Li, Li et al., 2021). This dense compact structure, naturally formed in several bakery products, such as bread and pasta, surrounds the starch granule, and can act as a secondary physical barrier limiting the contact between starch and digestive enzyme (Chen et al., 2019). Xu et al. (2021) observed that, by increasing the density of gluten/gluten hydrolysates, the rate and extent of starch digestion decreased in food models made with maize starch and gluten. Furthermore, peptides formed during fermentation might bind with starch, limiting starch accessibility, or directly inhibiting amylolytic enzymes (Liu et al., 2021; Lu et al., 2023; Xiong et al., 2023).

In **Chapter 4**, the role of gluten substitution on the textural properties and *in vitro* starch digestibility of bread samples made with coarse semolina was evaluated. The results indicated that the addition of gluten enhanced the textural properties of bread samples and led to an overall reduction in *in vitro* starch digestibility, compared to bread made solely with coarse semolina, regardless of the varying levels of dough hydration and mixing time. Similar findings were reported by Zeng et al. (2023), who observed an improvement in the textural quality of whole wheat breadcrumbs (decrease of hardness, and increase of volume and porosity), up to 20-30% of gluten substitution, and a reduction in *in vitro* starch digestibility when increasing the gluten content up to 50%. This effect may be attributed to the increased density of the gluten network surrounding the starch, which hinders enzymatic activity and reduces the accessibility of starch to digestive enzymes. Moreover, the gluten addition increased the cohesiveness of breadcrumbs, limiting their disintegration during digestion (see food matrix modification). However, **Chapter 5** presents a different trend when comparing the two bread samples made with fine semolina and different levels of gluten substitution (5% and 20%). Although the

glycemic response remained unchanged between the two samples, the bread made with 20% gluten substitution produced a higher amount of reducing sugars in the boli after mastication, leading to a greater release of insulin 30 minutes post-consumption, compared to bread made with 5% gluten. This difference could be due to the varying textural properties; bread with 20% gluten substitution was characterized by a more voluminous porous structure, facilitating the access of salivary α-amylase to starch granules, and thus leading to quicker starch hydrolysis, resulting in a higher sugar release in the oral phase. Conversely, the compact structure of the bread with 5% gluten substitution may have delayed starch hydrolysis at the oral level by restricting α-amylase access to starch. Therefore, the porous structure of bread made with 20% gluten substitution might have mitigated the effect of the gluten network density in reducing the starch digestibility. It would be insightful to isolate the impact of gluten density network from the structure of bread. Quantitative analysis of gluten density network is feasible through CLSM coupled with AngioTool64 software from the National Cancer Institute (Maryland, MD, USA). However, distinguishing between the effect of the gluten density network surrounding the starch granules, thereby limiting starch accessibility, and the impact of gluten in modulating the bread matrix is challenging.

6.2.3 Food matrix modification

The food texture, which is formed during processing, represents a macro barrier able to modulate the starch digestibility of starchy products (Gao et al., 2019). Moreover, several studies have shown that incorporating larger particles in bread can disrupt the formation of a well-structured elastic gluten network, leading to bread with a dense, hard, and not cohesive texture (Bressiani et al., 2017; Korompokis et al., 2021; Lin et al., 2020). In **Chapters 2** and **3**, the textural properties of durum wheat and rye bread produced with increasing flour particle sizes were characterized. We confirmed that bread made with medium (particle sizes >1000 μm and <1800 μm) and large (>1800 μm) flour was more prone to fracturing or crumbling than bread made with fine flour (<350 μm). During digestion, as detailed in **Chapter 3**, the physical breakdown of rye bread made with coarse flour was more extensive, leading to a larger surface area of the digesta and, consequently, increased accessibility for amylases. In contrast, rye bread made with fine flour, which maintained its structure, exhibited lower starch digestibility. Also in durum wheat bread, increasing the particle size of flour, the cohesiveness decreased, but the starch digestibility did not change. This difference between the two bread types is due to the functionality of durum wheat gluten, which leads to a denser network compared to the one formed in the rye bread, limiting the physical disintegration during digestion.

The physiological relevance of the disintegration rate of bolus during gastric digestion was well assessed by Vanhatalo et al. (2022) who performed an *in vivo* study in which, comparing the disintegration rate of durum wheat food products with different textures, demonstrated that a more cohesive structure led to bigger digesta particle after the gastric phase, and therefore, to a reduced starch digestibility. In **Chapter 4,** to deeply understand the effect of disintegration rate on starch digestibility, six bread samples with different cohesiveness were produced. Two different strategies were applied, the substitution

of 20% coarse semolina with vital gluten and the decrease in dough hydration. Modifications in dough hydration can modify the bread texture, producing denser structures that can lead to slower disintegration rates compared to traditional bread, consequently reducing starch digestibility (De La Hera et al., 2014; Lau et al., 2015; Martinez et al., 2018b). Furthermore, incorporating gluten into the dough alters the bread textural characteristics, decreasing hardness and chewiness while increasing springiness and cohesiveness (Zeng et al., 2023). Bread with lower hydration was found to be the hardest and the most fragile among the analyzed samples. Conversely, gluten-enriched bread samples generally exhibited better textural properties, such as lower hardness, higher cohesiveness, and greater volume, compared to those made solely with coarse semolina. These differences in textural characteristics of samples well mirrored the differences in starch digestibility. The hardest and the lowest bread volume showed the lowest value of RDS, instead the more porous bread showed higher RDS. Based on our results, during the first 20 min of intestinal digestion, the starch digestibility is mainly affected by the porosity of the crumb which leads to a more accessible starch in bread with a more aerated crumb structure. This relationship was further supported by a significant positive correlation (r= 0.854) between bread volume and RDS; the larger the bread volume, the higher the RDS value. However, the trend changes over the subsequent 100 minutes of intestinal digestion. During this phase, the samples with the highest cohesiveness proved to be the least digestible. Consequently, bread with 20% gluten substitution showed higher cohesiveness and exhibited lower SDS compared to bread samples made solely with coarse semolina. This reduced digestibility can be attributed to the preservation of bread structure during digestion, which potentially limits crumb disintegration and, hence, starch accessibility.

The relation between bread structure, physical disintegration, and starch hydrolysis was also well assessed in the *in vivo* study, reported

in **Chapter 5**. Despite the differences in textural properties among the three bread samples, no differences in glycemic response were observed. However, comparing the two bread samples made with fine semolina and different amounts of gluten (5 or 20%), the bread sample made with 5% elicited a lower amount of insulin 30 minutes after the sample consumption, than the sample produced with 20% gluten substitution, characterized by more expanse texture. Therefore, the porous structure of bread made with 20% gluten and fine semolina might have facilitated the access of salivary α-amylase to the starch granules, accelerating starch hydrolysis and increasing sugar release at the oral level. Conversely, the compact structure of bread made with 5% gluten may have delayed starch hydrolysis in the oral phase by restricting α-amylase access to starch, resulting in a lower insulin response (Eelderink et al., 2015).

In conclusion, the texture of bread had a key role in the modulation of starch hydrolysis. Generally, confirming what is already reported in literature, (De La Hera et al., 2014; Martínez et al., 2018), a compact dense structure reduces the starch digestibility, mainly in the first stage of digestion, leading to a lower RDS during the *in vitro* digestion and a lower release in reducing sugars after the mastication step.

6.3 Methodological considerations

This section describes the main features, challenges and limitations of the main methodologies used in this thesis.

6.3.1 Bread production

Throughout the thesis, several bread samples were produced to investigate the effect of cell wall integrity, gluten addition, and their interaction on textural properties and starch digestibility. In each experiment, bread samples were produced to have a complete experimental design useful for testing our hypothesis. However, in Chapters 4 and 5, due to technological limitations, not all the bread samples required for a complete experimental design could be produced. In Chapter 4, a bread sample prepared with only coarse semolina and mixed for 3.5 minutes would have been useful to isolate the effect of mixing time on bread made solely with coarse semolina in terms of increased cell wall porosity and consequent starch digestibility. However, as explained in Chapter 4, coarse semolina requires a long mixing time, up to 45 minutes, to completely develop its gluten network and, therefore, to form a structured bread texture. Consequently, producing bread with a short mixing time was not feasible. Moreover, in Chapter 5, the inclusion of bread samples made with 5% gluten and 95% coarse semolina would have been useful to in vivo test the effect of only coarse semolina, without the interference of 20% gluten substitution, on glycemic and insulinemic responses. However, also in this case, due to technological issues, this sample could not be produced.

6.3.2 Microscopic observations of cellular and bread structure

CLSM was effectively employed in the thesis (**Chapters 2** and **3**) to examine the presence of intact cell wall clusters in durum wheat and rye flours with varying particle sizes. Additionally, this method was used to observe the integrity of intact cells during the baking process, assessing the impact of processing on the cell structure. However, the methodology has some limitations, as it only indicates the presence or absence of intact cells without revealing changes in their permeability and porosity during baking. Previous research has explored cell wall porosity and permeability by monitoring the diffusion of enzyme-sized fluorescently labelled dextran using CLSM (Li, Gidley et al., 2019; Li, Zhang et al., 2019; Zahir et al., 2020), or through fluorescence recovery after photobleaching. Nevertheless, to assess cell porosity, an isolation step is required. Until now, such isolation has been performed only on intact wheat or sorghum, not on processed products like bread or digesta. However, due to the fragile structure of these materials post-baking, the isolation steps of the cells for CLSM analysis with labelled markers is quite challenging. Furthermore, isolating intact cells from flour would involve a pre-digestion step, which could alter the diffusion behavior of the probes by digesting cell walls and intracellular proteins, as noted by Zahir et al. (2020).

CLSM can also be used for visualizing the changes in the gluten network, protein density and how the flour particles affect protein development (Renzetti et al., 2021). The AngioTool64 software from the National Cancer Institute (Maryland, MD, USA) can be subsequently used to quantify the gluten density and protein branching. Moreover, by staining protein and starch with different dyes, it is possible to visualize the two nutrients together, monitoring their interaction. However, the sample preparation is quite challenging. This analysis is commonly performed on dough, freeze-

dried and thinly cut, and not on bread, limiting the knowledge about the gluten network after the baking process (Renzetti et al., 2021).
In conclusion, the porous fragile breadcrumb structure has limited the assessment of the cell wall porosity and the gluten density through the microscopic analysis. Further investigation on sample preparation protocol might overcome these technical issues.

6.3.3 *In vitro* starch digestibility

In vitro methods are utilized as preliminary assessments of the starch digestibility of various products, offering a less expensive and time-consuming alternative to *in vivo* tests (Englyst et al., 1992, Brodkorb et al., 2019). Throughout the thesis (**Chapters 2, 3** and **4**), the *in vitro* starch digestibility of bread samples was evaluated using Englyst's protocol (Englyst et al., 1992) because of its strong correlation with the glycemic index observed *in vivo* (Englyst et al., 1999). However, this protocol does not account for the oral phase of digestion, nor does it include the salivary α-amylase. This is because the quantities of enzymes used in Englyst's method are calculated to be in excess, ensuring they are not rate-limiting in the hydrolysis of starch to glucose (Englyst et al., 2000). Woolnough et al. (2010) found that the differences in starch digested during the oral phase are rapidly overwhelmed after the addition of pancreatin, which mitigated, at the end of the *in vitro* digestion, the differences in starch hydrolysis found after the oral phase. Other standardized methods, such as INFOGEST (Brodkorb et al., 2019), allow to skip the oral phase, considering its brief duration (typically around 3 minutes) and the subsequent gastric phase acidic pH (usually between 1.5 and 3), which rapidly inactivates salivary α-amylase (Woolnough et al., 2010). Despite this, results in **Chapter 5** of the thesis indicate that the amount of reducing sugars released in the bolus of study participants accounts for approximately 30% of the total starch in the analyzed bread. These released sugars are fundamental to take into account because they have a physiological relevance in insulin production and therefore the extent

of glycaemic response. Additionally, Freitas et al. (2022) highlighted the significant role of salivary α-amylase in *in vitro* starch digestion. Before its inactivation by the low gastric pH, salivary α-amylase can partially hydrolyze about 80% of the starch in bread and 30% in pasta. The inclusion of the oral phase during the *in vitro* starch digestibility is then crucial for enhancing the reliability between *in vitro* and *in vivo* data. Still, even more, this phase is fundamental for its physiological relevance in glucose metabolism. Different strategies have been employed to include the oral phase in the *in vitro* starch digestion. Some researchers have used a pre-mastication step before conducting *in vitro* digestion using several subjects, functioning as mastication devices. However, due to individual differences among subjects, this method may introduce systematic errors and imprecision in the *in vitro* digestion assessments. The individual oral processing behaviors can lead to significant variability in the properties of the bolus. Generally, boli of different dimensions can lead to differences in digestion rates, representing a real challenge in standardization (Gao et al., 2015, Gao et al., 2019). Other strategies include conducting an *in vivo* oral phase with a single subject (Freitas et al., 2018; Gao et al., 2019; Hutchings et al., 2012), but also this approach can lead to substantial error. In the context of the *in vivo* test analysis described in **Chapter 5**, we measured the activity of α-amylase in stimulated saliva for each study subject. Subjects were instructed to chew a piece of parafilm (5 × 10 cm, Parafilm M PM996) for 1 minute and collect the saliva in a pre-weighted Falcon conical centrifuge tube. The activity of amylase (U/mL) was then determined using a colorimetric saliva assay - the Ceralpha method (Megazyme, Bray, Ireland). The absorbance of the resulting solution, which is proportional to the amylase activity in the sample, was measured at 400 nm at 20 °C. This test was conducted twice for each subject under identical conditions. The test took place after two hours of fasting following the consumption of the glucose solution, and subjects were instructed to eat the same meal the night before each test. Despite these controlled conditions, the results of α-

amylase activity between the two replicates, as presented in Table 6.2, showed significant differences, with variations exceeding 100% for the same individual. This indicates that even using a single individual for testing can lead to significant errors due to the potential variability in α-amylase activity on different days.

Besides what is above discussed, physical disintegration, like the one obtained through mastication, also needs to be simulated. It can be achieved with a meat grinder or a blender, as reported by Gao et al. (2019). However, the ratio between bolus and saliva is difficult to standardize and must be adjusted according to each type of food to be tested to create boli with reliable physical characteristics (Gao et al., 2019).

In conclusion, as clearly shown in **Chapter 5**, the oral phase is fundamental to be carried out to increase the correlation with the *in vivo* data. However, at the same time, the test is quite challenging to standardize. Hypothetically, a good strategy to ensure the consistency of this step, is the use of pooled chewed samples in the *in vitro* digestion. The inter-individual differences might be mitigated, and the results may be more representative of a group of population.

Table 6.2: Means (U/mL), standard deviation and coefficient of variation of α-amylase activity of 16 study subjects.

	Mean (U/mL)	Standard deviation	Coefficient of variation (%)
S01	29.4	18.4	62.6
S02	49.7	1.03	2.1
S03	214.8	128.9	60.0
S04	79.9	55.9	70.0
S05	93.3	26.6	28.5
S06	42.5	8.1	19.0
S07	45.8	3.7	8.1
S08	22.5	8.2	36.4
S09	52.5	1.7	3.3
S10	56.4	44.6	79.1
S11	78.6	93.1	118.4
S12	100.9	17.8	17.6
S13	193.6	95.7	49.4
S14	48.8	20.1	41.2
S15	24.9	4.6	18.6
S16	141.3	182.0	128.8

6.3.4 *In vivo* study

The glycemic response in the human study was assessed following the ISO guidelines (ISO-26642, 2010). Referring to the methodology, a minimum number of 10 people is enough to determine the GI of a food product. In our study (**Chapter 5**), 16 volunteers were recruited to ensure the validity of the test. During the testing, blood glucose levels were measured using capillary blood finger pricks, a method which is sensitive and accurate enough to provide a good approximation of glucose concentration (Burt et al., 2013; Howard et al., 2020). Although the capillary finger stick procedure allows to measurement of blood glucose and insulin, using a venous catheter inserted in the participant's arm can expand the collection of data. For example, this sampling procedure allows the analysis of incretin hormones such as glucagon-like peptide-1 (GLP-1) and GIP, which are responsible for the production and release of insulin. This approach also enables blood sample collection over a longer period, ranging from 1 hour before the start of the test to 6 hours after bread consumption, as shown in the research by Eelderink et al. (2015, 2017). Furthermore, this blood sampling allows the use of stable isotopes (e.g., 13C) for the calculation of glucose kinetics (rate of appearance of exogenous glucose, endogenous glucose production, and glucose clearance rate) (Eelderink et al. 2015, 2017).

The oral processing analysis was conducted alongside the glycemic and insulinemic response evaluations to assess the effect of different bread textures on mastication behavior, glucose release, and bolus properties. Differences in bread textures have been shown to impact oral processing, mastication rate and bolus formation (Lau et al., 2015). These variations in oral behavior and bolus formation might partly explain the individual variations in glycemic responses to foods due to the different release of starch from the cellular matrix (Gao et al., 2015; Gao & Zhou, 2021; Chen et al., 2023). However, in our study, despite observing differences in textural properties, the mastication behavior

did not differ among the samples. This could be due to some limitations, important to acknowledge. Firstly, the oral processing analysis was based on the standardization of the weight of the bread bite rather than its volume. Previous research has shown that chewing behavior is mainly influenced by the volume of the product rather than its weight (Gavião et al., 2004; Mosca et al., 2022). Unfortunately, for the analyzed samples, providing a consistent volume among the samples, while maintaining a constant ratio between crumb and crust, was not feasible. For this reason, bread bites with the same weight were produced, ensuring the same ratio between crumb and crust. Another limitation was the relatively small number of subjects included in the mastication test. A larger group would have been beneficial for categorizing the study participants into fast and slow eaters, which would help in better understanding the relationship between chewing time, the release of reducing sugars during the mastication and insulin responses. Recent studies have shown that longer mastication duration and more extensive chewing of a food lead to higher saliva uptake in the bolus, which is associated with higher postprandial glucose and insulin response (Goh et al., 2021; Vanhatalo et al., 2022; Zhu et al., 2013). As mentioned in **Chapter 5**, insulin secretion was influenced by the amount of reducing sugars released during the oral phase. Hypothetically, longer mastication could lead to increased sugar production and, consequently, an increase in insulin production.

In summary, to enhance the robustness of the *in vivo* study, including the quantification of incretin hormones and assessing different oral processing behaviors would be beneficial for a deeper understanding of the mechanisms behind the reduced insulinemic responses in the developed bread.

6.4 Conclusion and future perspective

In response to the rising prevalence of T2D, CVD, stroke, and other non-communicable diseases linked to global increases in obesity, sedentary lifestyles, and consumption of energy-dense diets rich in highly digestible starchy foods, urgent measures are necessary. A strategy could be reducing GI of bread, which is a significant contributor to the glycemic load in Western diets (Rodríguez-Rejón et al., 2014).

This thesis aimed to investigate the feasibility of different physical barriers, such as cell walls, protein matrix, and food texture, and their interactions, on reducing the starch digestibility of different bread products. The utilization of flour containing clusters of intact cells was reported to be efficient in decreasing *in vivo* starch digestibility for minimally processed products such as porridge (Edwards et al., 2015), where starch granules not completely hydrolyzed were detected even after digestion in ileostomy volunteers. Our findings indicate that using only coarse flour (>1000 µm) to produce a more processed matrix like bread cannot effectively limit starch digestibility. This lack of effectiveness might be due to increased cell porosity during the baking process. In addition, several authors demonstrated that a compact crumb leads to a decrease in starch digestibility, thanks to less disintegration during the digestion process (Eelderink et al., 2015; De La Hera et al., 2014; Martínez et al., 2018). During the thesis, we observed that bread with a compact but fragile structure, such as that made with coarse semolina, easily disintegrated during digestion, increasing the contact surface between starch and enzyme, and resulted as highly digestible. This finding underlines the importance of cohesiveness, more than compactness, in modulating the starch digestibility of bread made with coarse semolina. To enhance bread

cohesiveness and the preservation of crumb structure during digestion process, gluten was added to the bread recipe.

In conclusion, based on the thesis results, only a holistic approach, combining the effect of coarse semolina, coupled with the addition of gluten, can only elicit a lower insulinemic peak, potentially mitigating the differences in glycemic response, and maintaining good textural properties. These thesis findings increased the overall knowledge on how to efficiently include coarse flour in bakery products to empower its ability in modulate starch digestibility.

However, further experiments are still needed to deeply understand the mechanism behind these results. As reported in Chapter 5, it is hypothesized that the lower insulinemic peak observed may be due to a low release of reducing sugars during the oral phase. The low glucose flow entering into duodenum may have potentially decreased the production of GIP, thereby influencing the insulin response. Quantifying this hormone, along with conducting additional experiments discussed in the previous paragraph, would be useful to deeply understand the mechanism behind these results and confirm our hypothesis.

As further perspective, the combination of gluten addition and coarse flour could be applied to other bread products such as rye bread. This bread is typically characterized by a dense hard structure. Previous research has shown that the unique texture of rye bread, coupled with its high fiber content, can effectively reduce the glycemic response compared to common wheat bread (Deleu et al., 2020; Jonsson et al., 2018; Rosén et al., 2009). However, this specific texture was not appreciated by the majority of the consumers, except people coming from northern European countries used to its peculiar texture. It has been observed that the addition of gluten can improve the textural properties of rye bread, leading to increased volume and reduced hardness. However, these textural modifications result in an increase of glycemic response (Nordlund et al., 2016), due to an improvement in porosity and therefore an increased accessibility to α-amylase.

Combining gluten addition with the use of coarse rye flour may be beneficial for the development of a rye bread product, characterized by appreciable textural properties and a potential low glycemic response.

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About the author

Marianna Tagliasco was born in Genoa (Italy) on December 30, 1994. In 2013, she moved from the sea to the Padan Plain to pursue her studies in Food Science and Technology at the University of Parma. After earning her bachelor's degree in 2016, she continued her studies in the same field at Parma University. In March 2018, she flew to West Lafayette, Indiana, USA, to conduct her master's thesis research in the laboratory of Prof Osvaldo Campanella at Purdue University. There, she began working on bakery products, focusing on the rheological properties of gluten-free dough. Upon receiving her master's degree, she started her career as a researcher at the University of Parma. During these initial months, she continued to work on bakery products, this time with a new focus that also included the nutritional aspects. Under the supervision of Prof Nicoletta Pellegrini, she started to research in the area of reducing the starch digestibility of bread products. After a few months, she moved to Wageningen University & Research in the Netherlands to continue her research in the Food Quality and Design group under the supervision of Prof Nicoletta Pellegrini, Prof Vincenzo Fogliano, Dr Edoardo Capuano, and Dr Stefano Renzetti. In 2020, she began her Ph.D. in Food Science and Health, focusing on the topic presented in her thesis. Her research was conducted between the Food Quality and Design group at Wageningen University & Research and the Department of Agriculture, Food, Environmental, and Animal Science at the

University of Udine. In 2022, she returned to Italy (Udine) to finalize her Ph.D.

During these three years, she supervised more than 15 MSc and Bachelor students at Wageningen University and Udine University. She attended several international and national conferences, workshops, and courses, delivering three oral presentations and presenting four posters on her research activity.

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